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DELIVERY SYSTEMS FOR BONE MORPHOGENETIC PROTEINS. A SUMMARY OF EXPERIMENTAL STUDIES IN PRIMATE MODELS

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ABSTRACT

The characterization and molecular cloning of the family of the bone morphogenetic proteins (BMPs) have laid the foundation for the cellular and molecular analysis of bone development and regeneration. A carrier substratum is required, however, to optimize osteogenic activity initiated by BMPs bound to the surface of the carrier. Native and recombinant human (rh) BMPs induce local endochondral bone formation in conjunction with the insoluble collagenous bone matrix, the inactive residue obtained after dissociative extraction of the matrix with chaotropic agents. While the cellular and molecular biology of BMPs and related members is advancing at a furious pace, progress in the formulation and implementation of novel delivery systems has been slow. The creation of inorganic nonimmunogenic carriers with defined geometries capable of delivering BMPs in the absence of the collagenous matrix is a crucial goal for skeletal reconstructionists and molecular biologists alike. Significant advances in skeletal reconstruction may be expected when novel carrier substrata are implemented for delivery of optimal doses of now available recombinant human BMPs.

KEY WORDS: BONE INDUCTION, BONE MORPHOGENETIC PROTEINS, EXTRACELLULAR MATRIX, COLLAGEN, OSTEOGENIN, POROUS HYDROXYAPATITE, DELIVERY SYSTEMS, PRIMATES

The cellular and molecular biology of bone development and regeneration is a central problem for skeletal reconstructionists. There is a direct relationship between growth and differentiation in early development and regeneration: postfetal osteogenesis, such as fracture repair, may be considered to recapitulate events that occur in the normal course of embryonic bone development (35). The reproducible initiation of bone differentiation in heterotopic sites by demineralized bone matrix represents evidence of a bone morphogenetic protein complex within the extracellular matrix of bone (37, 65, 67). The tissue response elicited by subcutaneous implantation of demineralized bone matrix is reminiscent of embryonic bone development, though a single cycle of endochondral bone formation is evident (34, 35).

TABLE 1

BMP and TGF-β families and bone induction: specificity and redundancy (modified from Reddi, 1992, ref 36).

BMP-2, BMP-4	+
BMP-3 (osteogenin)	+
BMP-5, BMP-6	+
OP-1 (BMP-7)	+
OP-2 (BMP-8)	?
TGF-β1, 2	-
ACTIVIN A	-
INHIBIN A	-
DPP	+
60A	+
Vg-1	?
GDF-1, 2, 3, 9, 10	?

Bioassay for in vivo cartilage and bone induction +, positive; -, negative; ?, unknown

The observation that demineralized bone matrix could be dissociatively extracted and inactivated with chaotropic agents (such as 4 M guanidine-HCl), and the osteogenic activity restored by reconstituting the inactive residue (mainly insoluble collagenous matrix) with solubilized protein fractions (53), confirmed the existence of bone morphogenetic proteins (54, 68, 69), and triggered a race for their purification (36, 41, 52, 66, 75, 81 for reviews).

The newly characterized family of bone morphogenetic proteins (BMPs) holds a realistic potential for the therapeutic reconstruction of the bone-bone marrow organ. Significant obstacles to their delivery, however, still limit the utilization of these molecular initiators as therapeutic agents.

The aim of this paper is to present a concise review of BMPs, and to focus selectively on different classes of carriers and substrata as delivery systems for them. Only the formulation and bioassay of osteogenic delivery systems with complete host acceptance and biological integration will established the potential therapeutic application of BMPs for craniofacial and orthopedic conditions.

THE BONE MORPHOGENETIC FAMILY AND RELATED MEMBE

The operational reconstitution of s protein fractions (the soluble signal inactive collagenous bone matrix soluble substratum) was a key e: that provided a bioassay for bona f tors of cartilage and bone different 53). It is noteworthy that by thems ther the solubilized proteins nor the collagenous matrix were active: h combination of the two restored activity in an extraskeletal bioass dents (36, 53). This functional bio vided the starting-point for the p of native bone morphogenetic (BMPs), which was followed by cloning and expression of the re human proteins (20, 76, 80). O BMPs, osteogenin, was isolated fr bone matrix by heparin-affinity c raphy, and purified to homogeneit troendosmotic elution after prepa dium dodecyl sulfate gel electroph The amino acid sequence of the t tides was determined and fou

unique (20). The amino acid sequence of osteogenin is identical to the sequence deduced from the cDNA clones of one of the recently characterized human bone morphogenetic proteins, BMP-3, cloned and expressed independently by Wozney et al. (80). Later, native osteogenin was also isolated and purified to apparent homogeneity from baboon bone matrix (46). Since the identification by molecular cloning approaches of human BMP-2a (now known as BMP-2), BMP-3 (osteogenin) and BMP-2b (now known as BMP-4) (80), several other BMPs, also called osteogenic proteins (OPs), have been identified, cloned, and expressed (5, 29, 56). The family of the BMPs/OPs is summarized in Table 1. Expression of the recombinant human proteins, with confirmation of their osteogenic activity in ectopic bioassay, has now been obtained for BMP-2 through BMP-7.

The BMPs show sequence homologies with members of the transforming growth factor-β (TGF-β) family (5, 29, 80), widely distributed in vertebrates and invertebrates (62). The BMPs including osteogenin (BMP-3), are related to developmentally critical regulatory genes such as the decapentaplegic (dpp) in Drosophila, which is implicated in dorsal-ventral specificaton (8), the 60A gene also in Drosophila, the expression of which suggests a role in embryonic mesoderm and ectoderm determination (79), and the vegetal (Vg-1) in Xenopus (78), with the Vg-1-related murine protein Vgr-1 (21). Other members of the TGF-β superfamily that share sequence homology with BMPs are the Mullerian inhibiting substance (MIS), which causes regression of the Mullerian duct during male development, and activins and inhibins, implicated in follicle-stimulating hormone release (60). More distantly related to these are the growth and differentiating factors (GDF-1, -2, -3, and -9) the biological functions of which have not yet been determined (17, 18, 24).

The fact that BMP-2 through -7 singly initiate endochondral bone formation in the ectopic bioassay raises an important question as to the biological relevance of this redundancy (36). The striking evolutionary conservation of the BMP genes indicates that they are critical in the normal development and function of animals. In addition to postfetal osteogenesis, the BMPs play multiple roles in embryonic development, including skeletogenesis, and may be involved in inductive events unrelated to bone induction that control pattern formation during embryonic organogenesis (16, 22, 23, 74). Moreover, the high levels of homology between dpp and 60A genes in Drosophila and human BMP-2, -4, and BMP-5, -6, and -7 respectively, raises further questions concerning the primordial role of BMPs during the emergence and development of invertebrates (36). Because of a possible possible evolutionary and functional conservation of these genes, they might have retained common developmental roles (79). Compelling evidence that these genes have been conserved for at least 600 million years is the exciting observation that recombinant Drosophila proteins, DPP and 60A, have the capacity to induce endochondral bone formation in mammals using the rat subcutaneous assay (58).

BMP-2 through -7 also share a limited homology with TGF-β molecules (5, 29, 80). The TGFs-β are themselves multifactorial regulators of cellular growth in developing systems, and can influence a wide variety of differentiation processes, including chondrogenesis, hematopoiesis and epithelial cell differentiation (62). While TGF-β molecules are most abundant in the extracellur matrix of bone (61), their in vivo role in bone regulation is not clear. The initiation of endochondral bone in the extraskeletal subcutaneous space in rodents permits the unambiguous bioassay of bone inductive molecules (36, 39). It is noteworthy that TGF-β_1 either purified from human platelets or expressed by recombinant techniques does not initiate endochondral bone formation in the in vivo bioassay (11, 54). Thus, the term initiation (or in-

duction) of bone formation should be reserved for a phenomenon elicited by BMPs and related molecules (36, 39). (Table 1). It is likely, however, that after the initiation of the first wave of bone differentiation by BMPs, the osteogenic cascade may be promoted and maintained by a variety of growth factors, including TGFβ (4, 39).

DELIVERY SYSTEMS FOR BONE MORPHOGENETIC PROTEINS

Osteogenesis involves the orderly migration, proliferation and differentiation of precursor osteogenic cells into functional osteoblasts. Vascular invasion, osteoblast differentiation and osteoid deposition ultimately result in the construction and remodeling of the cellular and extracellular matrix of bone. While the characterization of the BMP family may help to elucidate the molecular mechanisms underlying the initiation of bone formation, precursor osteogenic cells must attach to the solid substratum of extracellular matrix to spread, proliferate, and eventually differentiate (35).

Thus, a carrier substratum is required for optimal delivery of osteogenic activity initiated by BMPs bound to the surface of the carrier. The restoration of biological activity after dissociative extraction and reconstitution of BMPs with insoluble collagenous matrix indicates that components of the extracellular matrix of bone act as carriers for the functional expression of BMPs (53). Native and recombinant human (rh) BMPs induce local endochondral bone formation when reconstituted with the insoluble collagenous bone matrix, the inactive residue obtained after dissociative extraction of the bone matrix with 4 M guanidine-HCl (11, 20, 46, 56, 57, 76, 77) (Fig. 1).

Previous studies have shown that the insoluble collagenous carrier provides an optimal substratum for recruitment and anchorage of progenitor cells, and subsequent

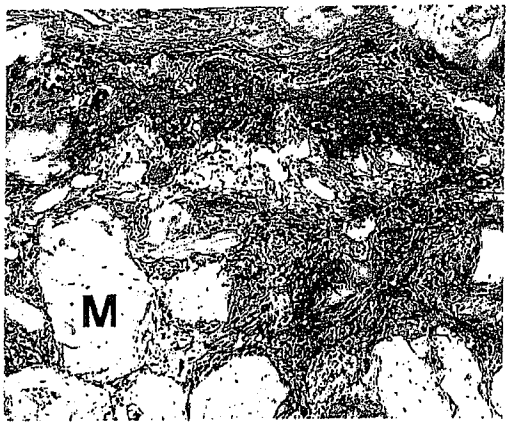


Fig. 1. Photomicrograph of a histological section of an implant of rat insoluble collagenous matrix (M) reconstituted with osteogenin (BMP-3) purified to homogeneity by electroendosmotic elution, and harvested on day 11 after subcutaneous implantation in a rat. Bone differentiation after vascular invasion and chondrolysis (toluidine blue, x145) (Source: Ripamonti et al., Matrix 12: 369, 1992, with permission).

proliferation and differentiation into osteoblasts (27, 32, 34). The importance of collagenous matrix in development and regeneration has been extensively demonstrated (35). In addition, this matrix may prevent premature diffusion at the site of surgical implantation (6, 39, 53), and protect BMPs from nonspecific proteolysis. Moreover, the collagenous carrier acts as a provisional substratum until replaced by new bone. RhBMPs, in conjunction with the insoluble collagenous carrier, have been shown to regenerate long bone defects in rats (83) and mandibular nonunions in dogs (64). The delivery of solubilized BMP fractions using purified collagenous preparations has also been reported in heterotopic and orthotopic sites (6, 26, 28).

It is noteworthy that angiogenic and bone morphogenetic proteins bind to extracellular matrix components. Osteogenin (BMP-3), rhBMP-4 and TGF-β_1 bind to type IV collagen (30, 31), which is an integral component

: basement membranes including the basal mina of blood vessels. Thus type IV colla-en and other extracellular matrix compo-ents (9, 73) may function as a delivery sys-m for both angiogenic and bone morpho-enetic proteins during bone development id regeneration (30, 31). Moreover, this ay link angiogenesis to osteogenesis, since pe IV collagen is a major constituent of iscular basement membranes. In recent ex-eriments, the discovery of the affinity of MP-3 for type IV collagen was used to test e osteogenic activity of BMP fractions ound to type IV collagen (19).

As a proteinaceous substratum, however, e use of the insoluble collagenous matrix id other collagen-based materials as deliv-y systems for BMPs is associated with a mber of drawbacks. These include inher-it weak mechanical performance, immuno-enic response, and potential transmission of ral antigens. In recent years, the quest for a itable implant to initiate and promote bone generation has led to the development of mposites of bone-inductive preparations id porous biomaterials as biological alter-atives to autogenous bone grafts. Thus, a tional approach to bone regeneration is the eation of osteogenic composites of porous omaterials and purified BMP preparations.

In recent experiments, it has been shown at the biological activity of native os-ogenin fractions could be restored and de-ered by a substratum with defined geome-y other than the insoluble collagenous ma-x (47). Adsorption of osteogenin (BMP-3) ictions onto porous hydroxyapatite re-lted in bone formation within the porous aces and in direct apposition to the inor-nic substratum when implanted ex-askeletally in rodents (47). In these experi-ents, the formation of bone within the cen-l porous spaces of the hydroxyapatite im-ants was noteworthy. This contrasted arkedly with results obtained using solubi-ed extracts of bone matrix delivered into ltered porous hydroxyapatites (15, 63). In both experiments after extraskeletal implantation bone formed only at the periphery of the hydroxyapatite implants. Moreover, bone formed only when protein fractions were delivered in conjunction with purified collagen (63).

Central to the preparation of osteogenic delivery systems for therapeutic use in humans is the demonstration of their biological activity in primates (7, 10, 12, 40). In recent years, evidence of heterotopic bone induction in primate species has been controversial. While Hosny and Sharawy (13) reported limited subcutaneous bone formation by induction in adult Rhesus monkeys, Aspenberg et al. (1) found no evidence of heterotopic bone formation after intramuscular implantation of demineralized bone matrix in adult squirrel monkeys. This has suggested that the concentration of BMPs within monkey bone matrix may not be sufficient to induce bone formation at intramuscular sites in adult monkeys (2).

The challenging problem of bone induction in primates has stimulated our laboratory to create animal models using adult baboons (Papio ursinus), which share similar, if not identical bone physiology and remodeling with man. Comparative static histomorphometric studies between iliac crest biopsies of humans and baboons showed a remarkable degree of similarity (59). This makes the adult male baboon ideally suited for the study of comparative bone physiology and repair with relevance to man.

The results of systematic studies in intramuscular sites of adult baboons unequivocally demonstrated bone formation by induction using bone matrix preparations and increasingly purified BMP fractions (40, 45, 46)(Fig. 2). Studies in other laboratories using different primate species have now incremented these observations in baboons, showing bone formation by induction in adult squirrel monkeys (Saimiri sciureus sciureus) and crab-eating monkeys (Macaca fascicularis) (3, 25). A systematic approach

Fig. 2. Photomicrograph of a histological section of an implant of baboon insoluble collagenous matrix reconstituted with osteogenin fractions and harvested on day 30 after intramuscular implantation in an adult baboon. Bone differentiation and mineralization of the newly formed bone around the invading capillaries (toluidine blue, undecalcified section at 5 μm after embedding in Historesin, x120).

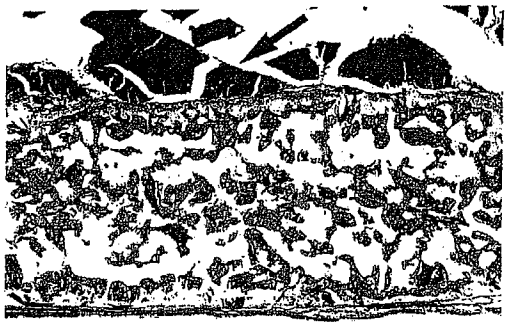
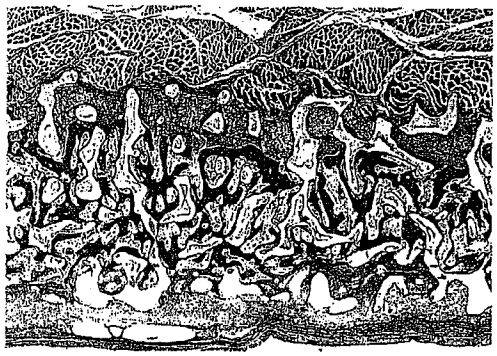


Fig. 3. Photomicrograph of a histological section prepared from a specimen of baboon insoluble collagenous matrix reconstituted with native osteogenin fractions 30 days after implantation in a calvarial defect in an adult baboon. Central region of the specimen: undecalcified sections, representing the sagittal diameter of the specimens, were cut at 7 μm and stained with the Goldner's trichrome. Extensive bone differentiation and formation of trabeculae of mineralized bone. Arrow indicates the temporalis muscle (x15) (Source: Ripamonti et al., Plast Reconstr Surg 91: 27, 1993, with permission).

was also taken to study the efficacy of different osteoconductive and osteoinductive substrata after orthotopic implantation in non-healing calvarial defects in adult baboons (42, 43). These experiments on bone regeneration in baboons have been undertaken in an effort to develop a reproducible primate model that would closely approximate calvarial repair in man, accelerating the pace of clinical trials (44). The comparatively large size of the adult male baboon calvaria allows the surgical preparation of four symmetrically located defects of 25 mm in diameter, with sufficient remaining intervening calvarial bone to limit the possible reduction of blood flow and vascular penetration within treated and untreated defects (42, 44). Using this primate model, it was shown that native osteogenin fractions completely regenerated calvarial defects in adult baboons 90 days after surgical implantation (46, 50). In these experiments, reconstitution of the insoluble collagenous carrier with osteogenin isolated from baboon bone matrix was a requirement for optimal delivery of biological activity (50) (Fig. 3).

Adsorption of osteogenin (BMP-3) and related BMPs onto hydroxyapatite gels is a chromatographic step for their purification (20, 39, 46, 56). This has resulted in the construction of delivery systems using porous hydroxyapatites activated by BMPs with osteogenic activity in extraskeletal sites in baboons (48). In recent experiments, composites of porous hydroxyapatite and native osteogenin fractions, adsorbed onto the hydroxyapatite, induced rapid bone differentiation in calvarial defects in adult baboons (49) (Fig. 4). By exploiting the principle of centripetal mesenchymal ingrowth (14), the porous hydroxyapatite appears to be well



g. 4. Photomicrograph of a histological section repared from a porous hydroxyapatite disc harested on day 30 after implantation in a calvarial defect of an adult baboon. Virgorous bone differentiaon within the porous spaces and in direct contact ith the hydroxyapatite after adsorption of osogenin fractions onto the substratum (toluidine ue, decalcified section at 5 μm, x15) (Source: Riamonti et al., Plast Reconstr Surg 90: 382, 1992, ith permission).

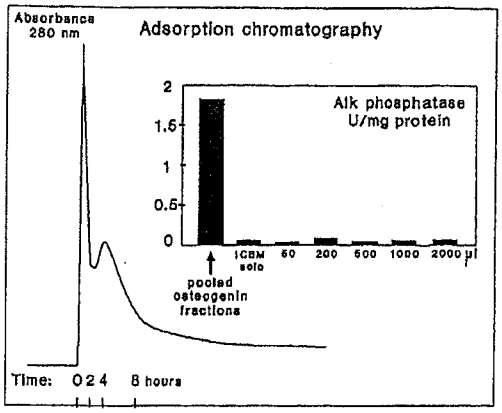


Fig. 5. Adsorption chromatography of baboon osteogenin fractions onto porous hydroxyapatite. Fractions in 4 M guanidine-HCl after Sephacryl S-200 gel filtration chromatography with biological activity in rats were pooled and exchanged with 5 mM HCl and loaded onto a chromatography column containing discs of porous hydroxyapatite 25 mm in diameter, and equilibrated in 5 mM HCl (20 ml of column volume). Adsorption of the material was monitored at an absorbance of 280 nm. After loading, the column was run in close circuit, continuously recirculating the hydroxyapatite eluate. The column was left to dry and the hydroxyapatite eluate (20 ml) collected and bioassayed for residual osteogenic activity. Before adsorption chromatography, aliquots of the loading material were analyzed electrophoretically and further bioassayed in the subcutaneous space of the rat. Inset: Alkaline phosphate activity on day 11 in implants of rat insoluble collagenous bone matrix (ICBM) reconstituted with pooled osteogenin fractions, ICBM without osteogenin as control, and ICBM reconstituted with 50, 200, 500, 1000 and 2000 μl of hydroxyapatite-unbound eluate. Ansence of biological activity in the hydroxyapatite eluate was confirmed by histologic examination. The electrophoretic profile on a 15% SDS-polyacrylamide silver stained gel under nonreducing conditions showed an apparent lack of protein in the hydroxyapatite eluate (not shown) (Source: Ripamonti et al., Biochem Biophys Res Commun 193: 509, 1993, with permission).

ılited for the formulation of delivery sys-
ems for BMPs. The porous substratum of
ydroxyapatite allows a spatially controlled
steogenesis, restricting bone differentiation
ically to surgical sites (47, 49). It is noteworıy that 1.5 μg of native osteogenin (BMP-3), urified to apparent homogeneity by electroıdosmotic elution, induced extensive bone ifferentiation when adsorbed onto the poous substratum, as evaluated histologically) days after calvarial implantation (46). Inerestingly, bone differentiation in porous ydroxyapatites in baboons takes place ithout an intervening chondrogenic phase 8, 49). This may have important therapeutic nplications when generation of cartilage is ot desirable.

In these studies of bone regeneration in ılvarial defects in primates, resorbable po-

rous substrata of calcium carbonate, with a remaining 2 μm surface coating of hydroxyapatite, performed poorly when compared with nonresorbable hydroxyapatite implants, even when pretreated with osteogenin fractions (49). Other resorbable compositions, however, have been found to deliver BMP activity effectively in heterotopic and orthotopic sites of mice and dogs (70, 71, 82).

In view of the potential therapeutic advantage of an inorganic, nonimmunogenic and biocompatible substratum in controlling the osteogenic activity of BMPs, recent experiments have exploited the affinity of native osteogenin for hydroxyapatite to construct an osteogenic delivery system after chromatographic adsorption onto a porous hydroxyapatite (51) (Fig. 5). Histologic examination of specimens implanted intramuscularly in baboons after chromatographic adsorption of osteogenin fractions showed extensive bone differentiation and prominent vascular invasion (Fig. 6). Specimen without prior adsorption of osteogenin showed penetration of fibrovascular tissue within the porous spaces but lack of bone differentiation (51). This new strategy may help to construct custom-made porous ceramics activated by BMPs, resulting in predictable bone formation for the treatment of human bone defects.

The geometry of the hydroxyapatite substratum exerts a profound effect on BMP-induced bone formation (47). After heterotopic implantation in both rodents and baboons, bone was never found in implants of granular hydroxyapatite. In contrast, bone differentiation was observed in blocks of hydroxyapatite, underscoring the importance of the geometry of the delivery system in bone induction by BMPs (47, 48, 72). The geometry of the substratum has been shown to influence profoundly the expression of the osteogenic phenotype in vivo (33, 38, 55).

Significantly, it has been demonstrated that the binding of one of the rhBMPs, BMP-4, prepared as described (11), is not affected

Fig. 6. In vivo bioassay in baboons. Hydroxyapatit specimens, harvested from the rectus abdominis were decalcified and double embedded in celloidi and paraffin wax. Serial sections, cut at 5 μm, wer stained with Goldner's trichrome. Extensive bone dif ferentiation on day 30 in a disc of porous hydroxya patite after chromatographic adsorption of baboo osteogenin fractions and intramuscular implantatio in an adult baboon (Source: Ripamonti et al., Biochen Biophys Res Commun 193: 509, 1993, with permis sion).

by the geometry of the hydroxyapatite sub stratum, since I^{125} radiolabelled rhBMP-4 binds equally well to hydroxyapatite sub strata in granular and block configuratio (47). These observations should be taken int consideration when planning the use o granular hydroxyapatites in reconstructiv craniofacial surgery.

FUTURE DIRECTIONS

We have previously proposed that the capac ity of mammalian BMPs to initiate a pro grammed cellular cascade that results in th induction of bone may be a functionally con served process utilized in embryonic devel opment and recapitulated in postfetal os teogenesis, and can be exploited for th therapeutic initiation of bone formation (46) The characterization and molecular cloning

of the family of the bone morphogenetic proteins and their interactions with extracellular matrix components has permitted the formulation of novel insights into the regulatory role of BMPs in osteogenesis (36, 75). From a therapeutic perspective, there is a need for focused research on the formulation of delivery systems for BMPs.

While the cellular and molecular biology of BMPs and related members is advancing at a furious pace, progress in the formulation and implementation of delivery systems has been slow. The formulation of non-immunogenic carrier substrata with defined geometries capable of delivering BMPs in the absence of collagenous matrix is a crucial goal for skeletal reconstructionists and molecular biologists alike. An ideal substratum acting as an osteogenic delivery system should be inorganic, nonimmunogenic, carvable and amenable to contouring for optimal adaptation to skeletal defects. Importantly, it should provide support and integration for the remaining periosteal tissues, and promote rapid vascular and mesenchymal invasion to be brought into contact with BMPs previously adsorbed onto the carrier. Lastly, it should remodel and resorb once the regenerative processes are well under way.

Finally, systematic studies will be required on the pharmacokinetics of rhBMPs in higher animals. This will promote the potential systemic therapeutic use of BMPs via noninvasive routes of delivery, desirable in systemic conditions such as osteoporosis.

The resolution at molecular level of some of the mechanisms regulating the initiation of bone formation will have a dramatic impact in craniofacial and orthopedic conditions. Significant advances in skeletal reconstruction may be expected when novel carrier substrata are implemented for delivery of optimal doses of now available recombinant human BMPs.

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Expedited Publication

Mandibular Reconstruction With a Recombinant Bone-Inducing Factor

Functional, Histologic, and Biomechanical Evaluation

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• Bone morphogenetic protein–2 (BMP-2) is a human recombinant bone-inducing factor that stimulates bone formation within 14 days. Twenty-six dogs underwent reconstruction of 3-cm full-thickness mandibular defects. After stabilizing the defects with stainless steel reconstruction plates, test implants composed of inactive dog bone matrix carrier and human recombinant BMP-2 were placed in defects of 12 animals (group 1). Control implants (carrier without BMP-2) were used in 10 animals (group 2), and no implants were placed in mandibular defects of four animals (group 3). Animals were killed at 3 and 6 months. The reconstructed segments were evaluated by roentgenography, analysis of functional stability, histology, histomorphometry, and analysis of biomechanical strength using three-point bend testing. In group 1, reconstruction plates were removed at 10 weeks because stiff, noncompressible mineralized bone formed across the defects, allowing the animals to chew a solid diet. The defects from groups 2 and 3 showed minimal, if any, bone formation and remained grossly unstable, prohibiting plate removal or advancement to a solid diet. Histomorphometric analysis at 6 months revealed that 68% of the group 1 implants were replaced by mineralized bone, whereas mineralized bone occupied less than 4% of the implants in groups 2 and 3. Biomechanical testing at 6 months revealed that the average bending strength of the reconstructed hemimandibles (expressed as a percentage of the contralateral hemimandible) was 27% for group 1 and 0% for group 2. The biomechanical strength of the defects reconstructed with BMP-2 increased significantly from 3 to 6 months and was related to degree of mineralization and thickness of bone bridging the defect.

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With the advent of mandibular reconstruction plates and microvascular transfer of osseomyocutaneous free flaps, surgeons have the capability of reconstructing large mandibular defects produced by cancer surgery or trauma. Titanium reconstruction plates and trays provide excellent temporary stability across mandibular defects, but lack of viable bone bridging the defect can result in exposure of the appliance, infection, bony erosion, mandibular instability, and, ultimately, facial deformity. Autologous bone grafts must be harvested from a donor site (iliac crest or rib) and usually provide insufficient bone that is prone to infection and/or resorption. Microsurgical transfers of free bone grafts with attached soft-tissue and blood vessels can close bony defects with an immediate source of blood supply to the graft. These techniques are time-consuming, can produce a great deal of morbidity, and can only be used by surgeons specially trained in microvascular tissue transfer. Furthermore, the bone provided is often limited in quantity, rectilinear, and not readily contoured to reconstruct curvilinear segments of the mandible (anterior arch). Even after reestablishment of mandibular continuity using presently accepted techniques, the majority of patients cannot wear dental appliances.[1-4] They gain a cosmetic benefit but with little improvement in masticatory function. Reconstructive surgeons should have at their disposal a bone substitute that would be reliable, biocompatible, easy to use, and long-lasting and that would restore mandibular continuity with little associated morbidity.

The ideal method for replacing lost mandibular bone would be to induce growth of host replacement bone that would bridge an entire defect by means of a bone-inducing implant. In 1965, Urist[5] reported incontrovertible evidence that rat bone demineralized in hydrochloric acid (0.6N) induced bone formation. Unfortunately, bone formation was variable and appeared to be dependent on several factors, including site of implantation and method of preparation. Demineralized bone has been shown to work well in fresh bony defects with abundant viable bone cells (osteoblasts and osteoclasts) and periosteum.[6-8] However, demineralized bone implants tend to resorb when used in nonbony sites (subcutaneous, intramuscular, or in a region with abundant scar tissue).[8,9] Furthermore, demineralized bone is an allograft, and despite sterilizing treatment with gamma irradiation using cobalt 60, one cannot rule out the possibility of transmitting infectious diseases.

The major problem with demineralized bone is that the amount of bone-inducing activity present in the implant is insufficient to induce bone formation in most nonbony sites.[9] Several proteins have been purified from bone matrix in an effort to extract the actual bone-inducing factor(s) present in demineralized bone. Urist et al[10] used a nondestructive method of differential solubilization to isolate a glycoprotein called *bone morphogenetic protein*. Even though this protein did stimulate bone formation in several animal models, it was not purified to

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homogeneity (not 100% pure) and was contaminated by other molecules that may have been responsible for the actual bone-inducing activity. Wang et al[11] have described a homogeneous human, recombinant bone-inducing factor that they called *bone morphogenetic protein–2* (BMP-2). The novel BMP-2 molecule is one of a group of seven polypeptides designated bone morphogenetic proteins 1 through 7.[12] Implantation of recombinant BMP-2 in the rat ectopic model induced bone formation by day 14.[11] This protein is a novel tissue factor that presumably transforms primitive mesenchymal cells into osteoblasts that form host bone.[11,12] Because this molecule is recombinant it (1) is free of contaminating proteins that may possess bone-inducing activity and (2) has the potential to be produced in large (commercial) quantities for clinical use. The fact that this molecule is human and recombinant distinguishes it from numerous partially purified bone-inducing preparations that are contaminated with other proteins and obtained from xenogeneic (bovine) bone. Partially purified xenogeneic preparations are not practical for clinical use because microgram quantities are purified from kilograms of bovine bone, making large-scale commercial production very costly and impractical.

In this study, dog bone matrix implants containing human recombinant BMP-2 were used to reconstruct 3-cm full-thickness segmental defects in the dog mandible. The purpose of this study was to evaluate the ability of the BMP-2 implants to (1) form host bone and restore mandibular continuity and (2) provide functional stability. We also sought to evaluate the tissue occupying the defects by histologic and histomorphometric analysis and quantitate the biomechanical strength of the reconstructed segments.

SUBJECTS AND METHODS

Preparation of the Implant

The carrier material used in this study was demineralized dog bone powder matrix that was treated with guanidinium chloride to extract any proteins that may possess inherent bone-inducing activity. Test implants consisted of 0.5 g of inactive dog bone powder matrix with 250 μg of human recombinant BMP-2 (10 μg of BMP-2 was added to every 20 mg of bone matrix) and 5 mmol/L of ε-aminocaproic acid. Control implants consisted of inactive dog bone matrix carrier without BMP-2 and 5 mmol/L of ε-aminocaproic acid. The implants were provided in a freeze-dried form (powder) that was sterilized with ethylene oxide and stored in a 5-mL syringe (Genetics Institute, Cambridge, Mass).

At the time of implantation, the freeze-dried implant material was mixed with 4 mL of fresh autologous blood and allowed to form a firm clot in the 5-mL syringe. The ε-aminocaproic acid was added to the implant to help stabilize the clot. After the clot set for 30 to 40 minutes, the implant material was allowed to slide out of the barrel of the syringe into the defect.

Table 1.—Stratification of the Study

Group	Type of Implant*	Time Interval, mo	No. of Dogs: Histomorphometry	No. of Dogs: Biomechanical Testing
1	Inactive dog bone matrix carrier *with* BMP-2 (test)	3	3	3
		6	3	3
2	Inactive dog bone matrix carrier *without* BMP-2 (control implant)	3	3	3
		6	2	2
3	No implant (control)	3	2	...
		6	2	...

*BMP-2 indicates bone morphogenetic protein-2.

Management of the Animals

Adult hounds weighing between 22 and 25 kg were studied according to a protocol approved by the Institutional Animal Care Committee of the University of Illinois College of Medicine, Chicago. Surgery was performed using endotracheal fluothane anesthesia after induction with thiamylal sodium (15 to 20 mg/kg intravenously).

Stratification of the Study

To test the validity and feasibility of this animal model and set up specific end points for analysis and criteria for mandibular stability, pilot data were collected from a four-animal pilot study. Two of these animals had BMP-2 implants, one had a control implant without BMP-2, and the last had no implant. These animals were killed at 3 to 12 months. These pilot data were also used to help stratify this study and determine the number of animals necessary to obtain statistically significant data.

The 26 animals used in this study (excluding four pilot animals) were divided into three groups (Table 1). Group 1 animals received test implants (carrier with BMP-2), and group 2 animals received control implants (carrier material without BMP-2). In group 3, no implant was placed into the defect. Twelve animals were in group 1; six were killed at 3 months and six at 6 months. Ten animals were in group 2; six were killed at 3 months and four at 6 months. Half of the animals killed in each of the time intervals were used for histologic and histomorphometric analysis, and the other half were used for biomechanical testing. Group 3 consisted of four animals; two were killed at 3 months and two at 6 months. All four of the group 3 animals were used for histologic and histomorphometric analysis. The stratification of the study is summarized in Table 1.

Preparation of the Cervical Feeding Esophagostomy

A cervical feeding esophagostomy was established at the time of reconstruction to permit early feeding of the animals. As a conduit off the distal cervical esophagus, the esophagostomy bypassed the oral cavity to prevent food particle contamination of the intraoral suture line and provided time for stabilization of the implant without pressure from mastication on the site of reconstruction.

An 8-cm midline incision was made in the lower aspect of the neck 5 to 6 cm above the sternal notch. The left sternomastoid muscle was dissected laterally, freeing it from the trachea and surrounding structures. The esophagus was identified immediately posterior to the trachea. After identifying the carotid artery, jugular vein, and vagus nerve, an 8-cm segment of esophagus was dissected from its fine areolar tissue attachments (Fig 1, top left). At this point, a 3-cm incision was made through the skin of the left lateral aspect of the neck, where a discrete division between the lateral neck muscles could be seen. The freed segment of esophagus was then pulled through the ostomy incision, and a posterior anchoring suture was tied to the muscularis layer of the esophagus and anchored deep into the dermis. The anchoring suture helped prevent the esophagus from pulling away from the ostomy site.

An incision was made through the muscularis layer of the esophagus parallel to the muscle fibers, and a 2-cm incision was made in the mucosa to enter the lumen (Fig 1, top right). The mucosa and muscularis were su-

tured with 3-0 polyglactin 910 (Vicryl) to the edges of the ostomy incision. Three other deep anchoring sutures fixed the adjacent esophagus to the surrounding dermis, encompassing enough muscularis to stabilize the esophagus to the lateral cervical skin (Fig 1, bottom). The midline incision was closed in two layers, leaving two Penrose drains in place for 48 hours. The esophagostomy was created immediately before the mandibular reconstruction.

This type of esophagostomy allowed the animals to be fed via a catheter inserted into the esophagus. However, the orientation of the esophagostomy as a conduit off the distal cervical esophagus also allowed the animals to take food orally with no obstruction, despite the presence of the esophagostomy.

Mandibular Reconstruction

After completion of the esophagostomy, a 6-cm skin incision was made parallel to the inferior border of the right mandibular body. After identifying the mandible and masseter muscle, an incision was made through the periosteum along the inferior border of the mandible. A periosteal elevator was used to elevate periosteum along both sides of the mandible, from the mental foramen to the mandibular angle posteriorly. Then, a 3-cm defect was marked on the mandible to include the first molar and fourth premolar teeth. To prevent disrupting the gingiva of surrounding teeth, a 3-0 polyglactin 910 suture was passed between the teeth, securing the gingiva to the adjacent teeth (third premolar and second molar) that were left in place. Taking care not to tear the gingival tissue, a periosteal elevator was used to elevate it from the teeth of the 3-cm segment that was to be resected.

A 10-hole stainless steel reconstruction plate (AO-ASIF, Synthes Maxillofacial, Paoli, Pa) was bent to match the contour of the buccal surface of the mandible. Using an air-driven drill and 2-mm drill bit, three holes were drilled on each side of the measured defect, taking care that the plate did not impinge on the mental nerve. Holes were then measured with a depth gauge and then tapped, and the plate was stabilized with six 2.7-mm cortical screws (Fig 2, top left). After removing the plate, a 3-cm full-thickness mandibular defect with teeth was created with a high-speed pneumatic saw (Fig 2, top right). The mandible was completely sectioned so that proximal and distal segments were separated by 3 cm. Electrocautery was used to stop bleeding from the ends of the defect. The reconstruction plate was reapplied to stabilize the mandible in its original preinjury orientation to ensure good dental occlusion (Fig 2, bottom left). Alveoloplasties were performed using a high-speed cutting burr to round off any sharp bony areas along the edges of the defect. The intraoral defect was closed with 3-0 polyglactin 910 mattress sutures to provide a water-tight seal.

The periosteum along both sides of the defect was elevated and resected to ensure that no periosteum was contacting the implant. Then, a thin areolar tissue flap was elevated along the buccal side of the defect and wrapped around the reconstruction plate to prevent bony ingrowth into the plate holes. This technique of enveloping the reconstruction plate also helped to facilitate plate removal at the designated time.

At this point, the defect was copiously irrigated with sterile saline. After achieving hemostasis, the test (carrier with BMP-2) or control (carrier without BMP-2) implants were allowed to slide out of the barrel of the 5-mL syringe into the mandibular defect (Fig 2, bottom right). The implants had the consistency of a firm fibrin clot and could be readily manipulated to occupy the contour of the defect. In the no-implant group, the mandibular defect was simply irrigated and left vacant. The wounds were closed in two layers. Postoperative roentgenograms were taken to document plate position, dental occlusion, and mandibular defect size and position.

Postoperative Care of the Animals

Animals were not allowed to take anything by mouth for 14 days. Intravenous catheters with heparin locks were left in place for 48 hours to allow infusion of daily fluid requirements and administration of cephazolin sodium (500 mg, intravenously every 12 hours for the first 2 days). On the third postoperative day, animals were given a liquid diet (Ensure, Ross Laboratories, Columbus, Ohio) administered with a 60-mL Toomey syringe and a Foley catheter (22F) inserted through the esophagostomy into the stomach (Fig 3). The animals were given three cans (250 mL per can) of Ensure twice a day, providing a total daily caloric intake of 5250 J (6.3 J/mL). Animals tolerated feedings well with no apparent discomfort and were examined once a day and weighed every other day to be sure they were not losing weight or becoming dehydrated.

On the 14th day postoperatively, esophagostomy feedings were stopped, and animals were allowed to take a soft diet orally. By this time, the intraoral suture line was completely healed, and the site of reconstruction had time to stabilize. At 10 weeks, the animals began a solid diet if specific criteria for mandibular stability were met.

Method of Analysis

To evaluate the ability of BMP-2 to form bone in a full-thickness canine segmental mandibular defect, the variables assessed were the following: (1) functional stability of the reconstructed segment; (2) contour, character, and dimensions of bone formation; (3) amount of mineralized bone formation (percentage area of defect tissue replaced by mineralized bone); and (4) biomechanical strength of the reconstructed segment compared with the contralateral nonoperated hemimandible and control implants (group 2). The immunogenicity of BMP-2 was evaluated to determine if the animals formed antibodies to human recombinant BMP-2.

Roentgenography and Physical Examination

The animals were sedated before the roentgenograms were administered, using atropine sulfate (0.05 mg/kg, intramuscularly) and 2.5% thiamylal sodium (1 mL/kg). Lateral roentgenograms of each mandible were taken with a collimator rotating anode tube-type machine (Universal Allied Imaging, Chicago, Ill) at settings of 300 mA, 0.05 second, and 59 kV peak immediately following reconstruction. Roentgenograms were also taken at monthly intervals and just before the animals were killed. While under sedation for the roentgenograms, the reconstructed segments were carefully examined and palpated to evaluate the contour and character (hardness and stiffness) of the implants. The contour of the implants was compared with the surrounding edges of the mandibular defect. Additional roentgenograms and precise mandibular measurements were taken of the harvested reconstructed segments of the mandible when the animals were killed.

Analysis of Functional Stability

Animals underwent removal of the reconstruction plates at 10 weeks if the defects appeared stable by roentgenographic evaluation and physical examination. Roentgenographic criteria for stability of the reconstructed segments included (1) evidence of mineralized bone formation across the entire defect and (2) evidence of integration of the new bone with the edges of the defect. Stability of the reconstructed segment was also based on physical examination, which revealed palpable bone formation across the entire defect. Specific criteria indicating stability of the new bone included the following: (1) noncompressibility, (2) stiffness (enough to resist bending), and (3) approximation of the dimensions of the defect. These criteria are subjective in nature; however, information obtained from the pilot studies indicated that the degree of bone formation at 10 weeks in animals with BMP-2 implants was sufficient to support mastication in the absence of the reconstruction plate if these criteria were met.

After plate removal, animals began a solid diet until the time when the animals were killed. The ability of the animals to tolerate a

solid diet was evaluated by the following criteria: (1) observation of their eating habits, (2) amount of food intake, and (3) weight loss. Plate removal was not attempted in any animals in group 2 (control implants without BMP-2) or group 3 (no implants), because pilot studies revealed gross instability of the reconstructed segment when roentgenographic evaluation and physical examination revealed no evidence of bone formation across the defect. In these animals, the plates were left in place until the animals were killed at 3 or 6 months after the reconstruction.

Immunoreactivity to BMP-2

Immunoreactivity to human recombinant BMP-2 was assayed in all animals used in this study. Serum was prepared from blood drawn just before sacrifice and stored at −80°C. Immunoreactivity to human recombinant BMP-2 was determined by immunoblot and visualized (iodine 125-labeled Protein-A, New England Nuclear, Boston, Mass).[13]

Harvesting Specimens

After 3 or 6 months, the animals were killed, and the entire mandible was harvested from each animal, leaving the gingival mucosa intact around the site of the reconstructed segment. At the midpoint of the reconstructed segments, height measurements were made in the vertical plane, and width measurements were made in the transverse plane. In the contralateral nonoperated hemimandible, height measurements were made at the first molar from the lower border of the mandible to the superior margin of the alveolar process. Width measurements were made from the lingual cortex to the buccal cortex at the widest point of the mandible in the region of the first molar. The mean measurements of the reconstructed segments were expressed as a percentage of the mean measurements of the contralateral nonoperated hemimandible. After photographic and roentgenographic documentation, the specimens used for histologic and histomorphometric analysis were cut to a 6.5-cm segment, leaving at least a 1.5-cm cuff of normal mandible on both sides of the reconstructed defect. Mandibles submitted for biomechanical testing were cut into longer hemimandibular segments that allowed insertion into the apparatus used for three-point bend testing. Implants harvested for biomechanical testing were packaged and frozen so all could be tested at the completion of the study.

Histologic and Histomorphometric Analysis

Specimens submitted for histologic and histomorphometric analysis were fixed in phosphate-buffered formaldehyde and embedded in methylmethacrylate. After the embedded specimens were allowed to cure, the blocks were cut as follows. An initial cut was made longitudinally (sagittal plane) close to the center of the specimen with an Isomet precision saw (Buehler, Lake Bluff, Ill). To reach the midsagittal plane, further longitudinal sections were taken across the entire length of the specimen (thickness, 10 μm) with a Polycut-S microtome (Reichert-Jung, Nussloch, Germany). Final sections were cut (thickness, 5 μm) and then treated according to the method of Schenk et al[14] and stained with a modified von Kossa's stain containing silver nitrate, basic fuchsin, and toluidine blue O. The silver nitrate stained all mineralized bone black, allowing quantitation of the extent of mineralization. Using an Optimax 5 image analyzer (Analytical Measuring System Ltd, Cambridge, England), the area density of mineralized tissue was measured by determining the percentage of mineralized area per standard tissue area. Since area density equals volume density (assuming new bone is evenly distributed in the defect), the percentage volume of the tissue occupying the defect that had formed mineralized bone could be determined.[15] After determining the means and SDs, statistical analysis was performed to compare the percentage area of mineralized bone in groups 1, 2, and 3. Analysis of variance and Tukey's multiple comparisons method were used to determine if the differences between the group means were significant statistically with respect to time effects or treatment effects.

Biomechanical Testing

Three-point bend testing was performed on the reconstructed hemimandible and the contralateral (nonoperated side) hemimandible of all animals designated for biomechanical testing. To compare the reconstructed hemimandible with the contralateral hemimandible, a control experiment was performed to validate that such a comparison could be made. The right and left hemimandibles of eight normal dogs underwent three-point bend testing to determine the variability between both sides. A classic analysis of means was used to analyze the mean ratios of the failure moments between the right and left hemimandibles (both sides unoperated).

Hemimandibles were stripped of soft tissues and placed within the three-point bending system. The central load point was oriented between the fourth premolar and the first molar (Fig 4) or at the midpoint of the reconstructed segment. The other two load points were set 5.4 cm from the central load point. Mandibles were tested to failure in three-point bending, with the lingual surface in tension, using an Instron 1331 servohydraulic materials-testing system (Instron, Canton, Mass). A stroke rate of 1 mm/min was applied to a maximum central load point displacement of 10 mm. Moment and displacement data were recorded using notebook software (Labtech Notebook, Laboratory Technologies Corp, Wilmington, Mass) on a personal computer using an 80386 microprocessor (Intel, Santa Clara, Calif). Failure was defined as the maximum moment recorded during the test. Equivalent bending rigidity was determined for the initial linear segment of the data (if a linear region existed). After determining the maximum moment of the reconstructed segment, the bending strength was also expressed as the percentage of the maximum moment of the contralateral hemimandible.

Statistical analysis was performed on the maximum moment values (N − m) for groups 1 and 2. After determining the means and SDs for the maximum moments of the reconstructed segments and percentage of the maximum moment of the contralateral hemimandible, analysis of variance and Tukey's methods of multiple comparisons were used to determine if the time effects and treatment effects between group means were significant statistically.

In another experiment, 20 normal unoperated hemimandibles were harvested and used to evaluate the biomechanical strength of mandibles reconstructed with only a stainless steel or titanium reconstruction plate. Employing the same technique used on the study animals, 10-hole reconstruction plates (AO-ASIF, Synthes Maxillofacial, Paoli, Pa) were contoured to the buccal surface of the body of the harvested hemimandibles. Plates were fixed to the hemimandibles using six 2.7-mm cortical screws, with three screws on each side of the proposed 3-cm full-thickness mandibular defect. The plates were removed and the hemimandibles were sectioned, leaving proximal and distal segments separated by 3 cm. Plates were reapplied to stabilize the hemimandibles in their original preinjury orientation. Ten hemimandibles were reconstructed with stainless steel plates and screws, and 10 were reconstructed with titanium plates and screws. Three-point bend testing was performed with the central load point in the middle of the plate. The other two load points were set 5.4 cm from the central load point. The maximum moment was determined using a stroke rate of 1 mm/min with a maximum central load point displacement of 10 mm. After determining means and SDs, the mean maximum moment of the hemimandibles reconstructed with BMP-2 was compared with those bridged by only a reconstruction plate. Analysis of variance and Tukey's method of multiple comparisons were used to determine if statistically significant differences in biomechanical strength existed between mandibles reconstructed

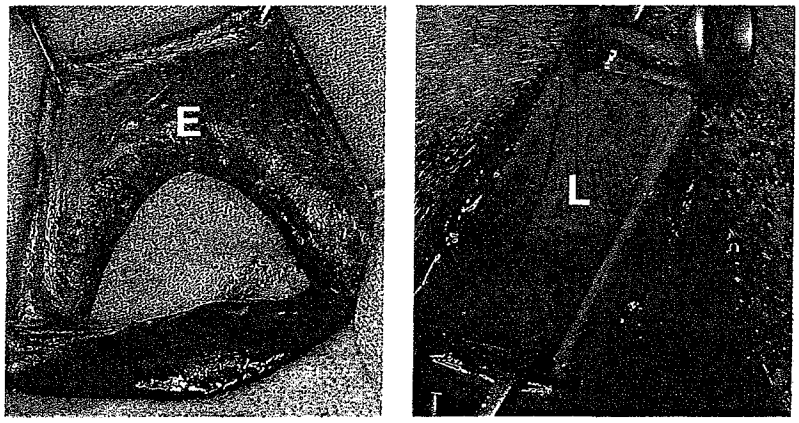


Fig 1.—Cervical feeding esophagostomy. Top left, The esophagus was identified posterior to the trachea and an 8-cm segment was dissected free from surrounding tissues. E indicates esophagus. Top right, After the esophagus was brought through a small lateral neck incision (site of esophagostomy), an incision was made in the lateral wall of the esophagus, and the lumen was identified. L indicates lumen of esophagus. Bottom, Esophagostomy shown as a conduit off the distal cervical esophagus, permitting unobstructed passage of food from above. Note how the anchoring sutures fix the esophagostomy to the lateral cervical wall. E indicates esophagus; arrows, esophagostomy suture line.

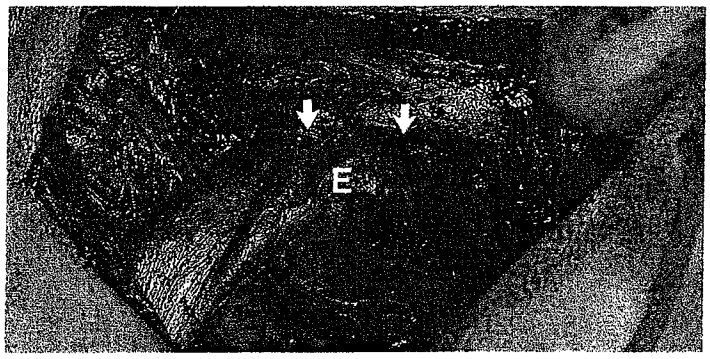
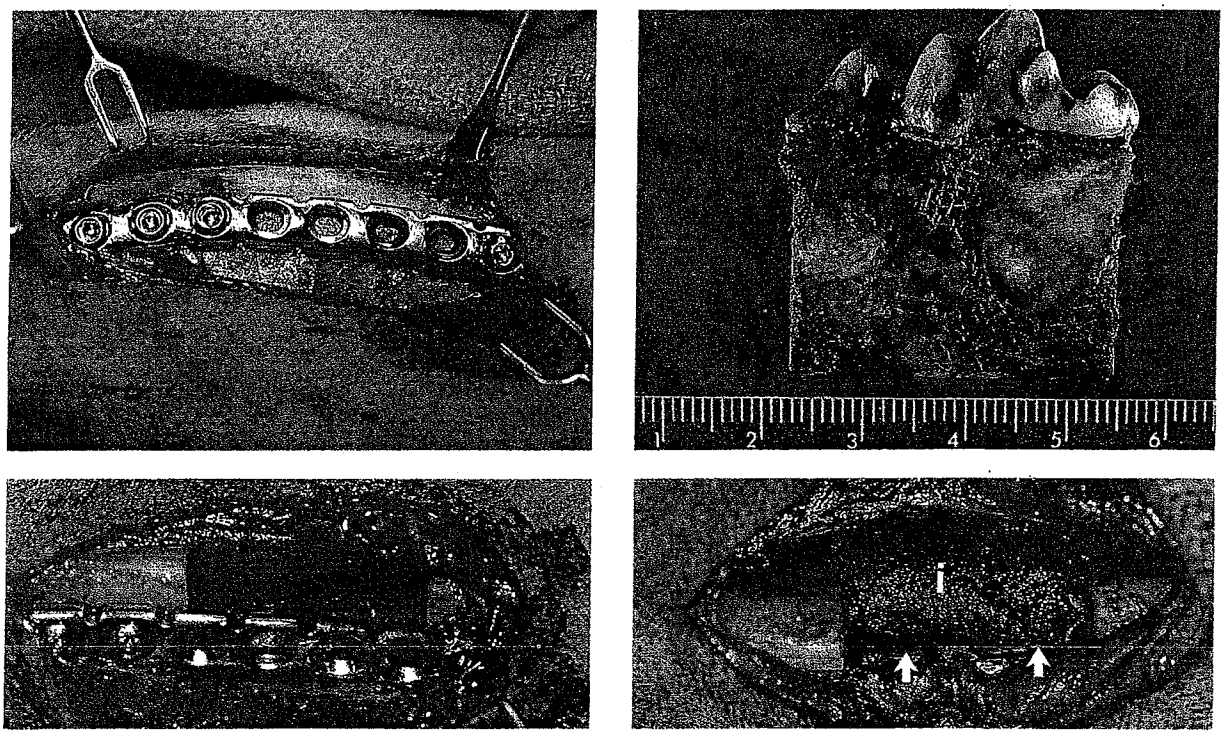


Fig 2.—Mandibular reconstruction. Top left, Stainless steel reconstruction plate contoured and fixed to the buccal surface of the mandible with three cortical screws on each side of the proposed 3-cm defect. Top right, Full-thickness (3-cm) mandibular segment including fourth premolar and first molar. Bottom left, Reconstruction plate reapplied to stabilize mandible in its original preinjury orientation. Bottom right, Implant was mixed with autologous blood and allowed to clot. The implant was then placed in the defect and molded to contact the edges of the bony defect. Note how a thin layer of areolar tissue (arrows) has been used to cover the plate. The letter "i" indicates implant.



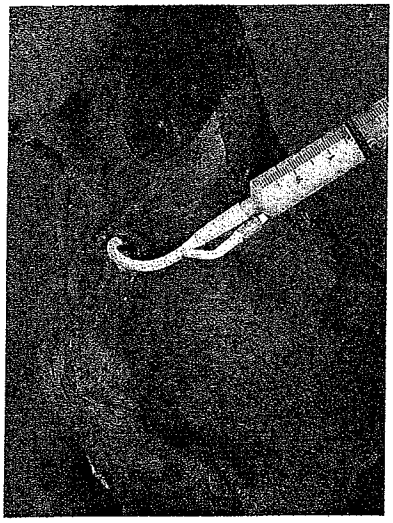


Fig 3.—Esophagostomy tube feedings. Foley catheter and 60-mL syringe were used to instill feedings through the esophagostomy directly into the stomach.

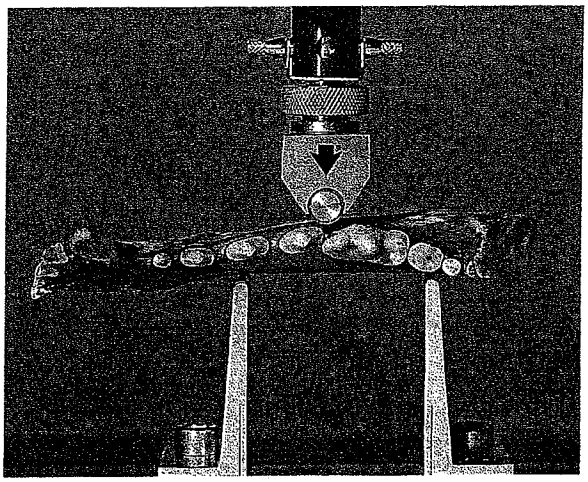
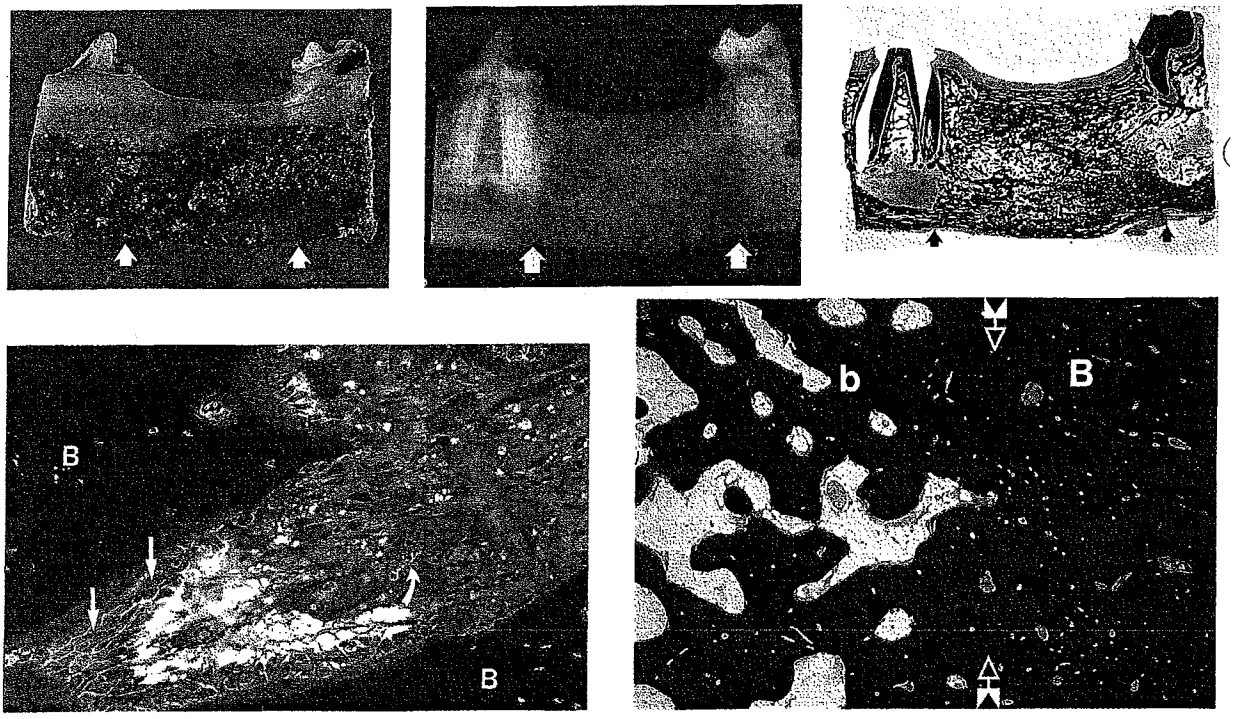


Fig 4.—Control, nonoperated hemimandible loaded into the three-point bending apparatus. Arrow indicates direction of the load.

Fig 5.—Group 1 (3-month specimen). Top left, Harvested reconstructed segment shows bone formation across the 3-cm defect. Arrows point to edges of the defect. Top center, Roentgenogram demonstrating fully mineralized bone growth across the defect. Arrows mark edges of defect. Top right, Histologic section demonstrating mineralized bone extending across the defect. Mineralized bone is represented by the black-stained area and occupied 52% of the tissue within this defect. Arrows mark edges of the defect (modified von Kossa's stain, original magnification ×0.5). Bottom left, Active bone formation with plump osteoblasts (arrows), osteoid seams, and blood vessels (curved arrows). B indicates mineralized bone (modified von Kossa's stain, original magnification ×250). Bottom right, Interface between new bone on the left (b) and old bone on the right (B). Arrows point to the region of transition from the new bone to the old bone. Note the excellent degree of integration of the new bone (modified von Kossa's stain, original magnification ×25).



with BMP-2 and mandibles reconstructed with only a metal plate.

RESULTS

The results of the pilot study were not included in the compilation of the data of the 26 animals stratified into groups 1, 2, and 3.

Complications

All animals survived the duration of the study, tolerating 14 days of esophagostomy feedings with less than a 5% weight loss, which was rapidly corrected once the animals were started on a soft diet. The animals actually learned to anticipate their tube feedings, sitting still and cooperating throughout the entire feeding process. After completion of esophagostomy feedings, the stoma gradually constricted to a small opening within 6 weeks. In three animals, saliva and orally ingested food leaked out of the esophagostomy, requiring insertion of several sutures to close the stoma. Despite the presence of the esophagostomy, no obstruction of the passage of solid food to the stomach could be found.

Seromas of the lower part of the neck developed in six animals in a region of cervical skin redundancy. Four of these seromas resolved after aspiration. Seromas developed in two animals, resulting in partial dehiscence of the esophagostomy suture line. In both of these cases, a minor surgical procedure was required to resuture the esophagus to the skin.

Infections did not develop in any of the group 1 animals (test implants, carrier with BMP-2). However, localized infections developed in three animals from group 2 (control implant, carrier without BMP-2) at the implant site. These infections cleared with a 10-day course of antibiotic therapy (cephalexin, orally). Small (1- to 2-mm), nondraining fistulas developed in two group 2 animals at the site of the intraoral suture line that persisted until the animals were killed. These animals remained asymptomatic and did not require any surgical management.

Roentgenographic Analysis and Physical Examination

Roentgenograms taken immediately after surgery documented good plate position, alignment of the mandible, dental occlusion, and defect size. In group 1 animals (test implants with BMP-2), earliest bone formation across the defect was detected roentgenographically at 21 days. Later roentgenograms revealed increasing bone density with evenly distributed bone formation occurring throughout the entire defect. Palpation of the reconstructed segments as early as 5 weeks revealed stiff, noncompressible bone bridging the defect, with mandibular height along the vertical axis of the mandible that compared favorably with normal bone surrounding the mandibular defect. The reconstructed segments demonstrated a slight decrease in width along the transverse axis when compared with normal bone surrounding the mandibular defect. The mucosal suture lines were healed well in all animals in group 1.

Roentgenographic evaluation of group 2 animals (control implants without BMP-2) revealed minimal, if any, bone formation across the defect at 3 and 6 months, with a large gap between both segments. Analysis of group 3 animals (no implant) at 3 and 6 months revealed no bone formation across the defects.

Palpation of the reconstructed segments of the animals in groups 2 and 3 revealed an empty defect bridged by a reconstruction plate with no palpable bone formation. The sites of the mucosal defects were well healed, except for the two animals in group 2 with small nondraining fistulas.

Functional Stability of the Reconstructed Segments

Early removal of the stainless steel reconstruction plates allowed evaluation of the functional stability of the reconstructed segment of the mandible. Plates were removed at 10 weeks in all animals in group 1 because new mineralized, stiff, noncompressible bone formed across the defects and appeared to be integrated with the edges of the defect. All of these animals tolerated a solid pellet diet, chewing their food without difficulty, evidence of discomfort, or weight loss. Plates were also removed at 10 weeks in group 1 animals (BMP-2 implants) that were killed at 6 months. These animals also tolerated a solid pellet diet without problems.

Plate removal was not attempted in any of the animals in group 2 (control implant) or group 3 (no implant) because of obvious instability of the reconstructed segments that showed minimal or no bone formation across the defects. Animals in groups 2 and 3 remained on a soft diet throughout the course of the study.

Immunoreactivity to BMP-2

No antibodies to human recombinant BMP-2 were detected in any of the animals by immunoblot using 0.5 μg of BMP-2 and a 1:100 dilution of serum.

Analysis of Harvested Specimens

Group 1 (test implants with BMP-2) specimens revealed extensive bone formation across the entire defect at 3 and 6 months. The contour of the reconstructed segments approximated the contour of the surrounding mandible with no evidence of bone growth beyond the margins of the defect (Fig 5, top left). Mandibular measurements (mean) revealed that height along the vertical axis of the reconstructed segments approximated 86% of the height of the contralateral, nonoperated hemimandible. However, the mean width of the reconstructed segments along the transverse axis approximated 62% of the corresponding measurements of the contralateral nonoperated hemimandibles.

Roentgenographic examination of the 3-month group 1 animals revealed mineralized bone formation across the entire 3-cm defect (Fig 5, top center). Histologic and histomorphometric analysis revealed that 50% of the tissue occupying the defects was mineralized and that this bone was almost as dense as the normal bone surrounding the mandibular defect (Fig 5, top right). The surfaces of the new bone were lined with osteoid seams and plump osteoblasts, indicating active bone formation (Fig 5, bottom left). Among the 3-month group 1 animals, occasional islands of residual bone matrix carrier material were seen. Good fusion between the old and new bone was observed so that the interface between the edges of defect and implant was difficult to identify (Fig 5, bottom right).

Group 1 specimens harvested at

6 months showed bone formation across the entire defect with excellent contour and mineralization. Histologic and histomorphometric analysis revealed that 68% of the tissue occupying the defects was mineralized with minimal, if any, evidence of residual carrier material (Fig 6, left). The bone demonstrated a normal-appearing lacunar pattern with no evidence of bone resorption as determined by the absence of osteoclasts (Fig 6, right). The newly formed bone appeared normal, making it difficult to identify the point of transition from BMP-2 implant to normal bone surrounding the defect.

Group 2 specimens (control implants without BMP-2) revealed minimal, if any, bone formation with some evidence of bone resorption where the reconstruction plates eroded into the bone surrounding the mandibular defect (Fig 7, top left and right). When the animals were killed, it was noted that the reconstruction plates were loose in four animals in group 2. These plates may have loosened because of bony erosion under the reconstruction plates with subsequent loosening of the screws. At 3 months, histologic investigation revealed fibrous scar tissue (callous) bridging the defect with no bone and relatively large amounts of residual nonviable bone matrix (Fig 7, bottom left and right). At 6 months, fibrous scar tissue (callous) bridged the defect, and any bone that did form within the defect was spotty, irregular, and formed in the region of the approximated gingival mucosa where small amounts of periosteum may have been left behind (Fig 8). Only 4% of the tissue occupying these defects was replaced by mineralized bone with very few active osteoblasts, flat lining cells, and osteoclasts, indicating inactivity of bone-forming cells.

Histomorphometric examination of group 3 specimens (no implant) at 3 and 6 months revealed fibrous scar tissue with no bone formation. The margins of the bony defect showed flat lining cells indicating low bone cell activity. Of the animals in group 3, two had loosened reconstruction plates, also probably due to bone erosion under the plates. In no control animal was there a complete bridge of new bone across the 3-cm mandibular defect.

Table 2 shows the percentage area (mean) of the tissue occupying the defects in each group that was mineralized. The differences in mineralization between group 1 and the control groups (groups 2 and 3) were statistically significant at $P<.001$.

Biomechanical Testing

Three-point bend testing of the eight paired, nonoperated normal mandibles revealed that the mean ratio (right to left) for the maximum moments of both control hemimandibles (both sides nonoperated) was 1.057 (SD = 0.116). Using a classic analysis of means, this mean value is not different from 1.0 ($P=.243$). These data verify that the bending strength of a reconstructed hemimandible can reliably be compared with the contralateral, nonoperated hemimandible because the preinjury differences between both sides of the mandible were not significant statistically.

Results of the three-point bend testing performed on the specimens in groups 1 and 2 are shown in Table 3. The specimens in group 1 demonstrated failure in three-point bending at a mean maximum moment of 7.4 N-m and 22.6 N-m for 3- and 6-month specimens, respectively. These values were 9% and 27% of maximum moment values of the contralateral (nonoperated) hemimandible for the 3- and 6-month specimens, respectively. This average time effect noted between 3 and 6 months across group 1 was significant statistically at $P=.015$. The group 1 reconstructed segments demonstrated a nonlinear relationship between moment and displacement, which indicates plasticity of the new bone. However, the contralateral nonoperated hemimandibles showed a clear linear relationship between initial moment vs displacement. Maximum moment values for the group 2 specimens were 0.0 N-m for the 3- and 6-month specimens. These values were nondetectable due to absence of bone formation. The average treatment effect noted between groups 1 and 2 across both time intervals was significantly different statistically at $P=.004$. Biomechanical testing was not performed on any of the group 3 specimens, which also showed no evidence of bone formation and would probably yield nondetectable bending strengths.

Mean maximum moment values for the mandibular defects reconstructed with stainless steel and titanium reconstruction plates alone was 14.9 N-m and 17.3 N-m, respectively. The mean maximum moment of the 6-month group 1 specimens (carrier with BMP-2) (22.6 N-m) was significantly greater than the mean maximum moment of hemimandibles reconstructed with stainless steel or titanium reconstruction plates at $P=.017$ and $P=.093$, respectively. Expressed as a percentage of the 6-month group 1 specimens, the mean maximum moment of the stainless steel and titanium plate reconstructions were 66% and 76%, respectively.

COMMENT

In this study, BMP-2 bone-inducing implants were used to reconstruct full-thickness segmental mandibular defects in dogs. The purpose of this study was to determine if BMP-2 could induce host bone formation across a 3-cm mandibular defect and provide sufficient functional stability to allow the animals to chew a solid diet. Biomechanical testing was performed to quantitate the bending strength of the reconstructed segments. Full-thickness (3-cm) mandibular defects in the body of the canine mandible were used because this size defect in 22- to 25-kg hounds did not heal spontaneously if the periosteum around the defect was resected (pilot study). With no evidence of significant bone formation in the absence of periosteum, 3-cm full-thickness defects in this size animal can be considered a critical size defect.[15] Furthermore, a defect at this location will readily accept a contoured reconstruction plate. The plate provided temporary rigid fixation, stabilizing the defect and aligning dental occlusion. With stabilization of the mandible, test or control implants could be placed in the defects without disrupting the preinjury occlusal orientation.

In previous studies on mandibular reconstruction using a canine model, teeth at the site of mandibular resection were extracted 4 to 16 weeks before the actual reconstructive procedure.[17,18] Extraction of teeth was performed beforehand to permit closure of

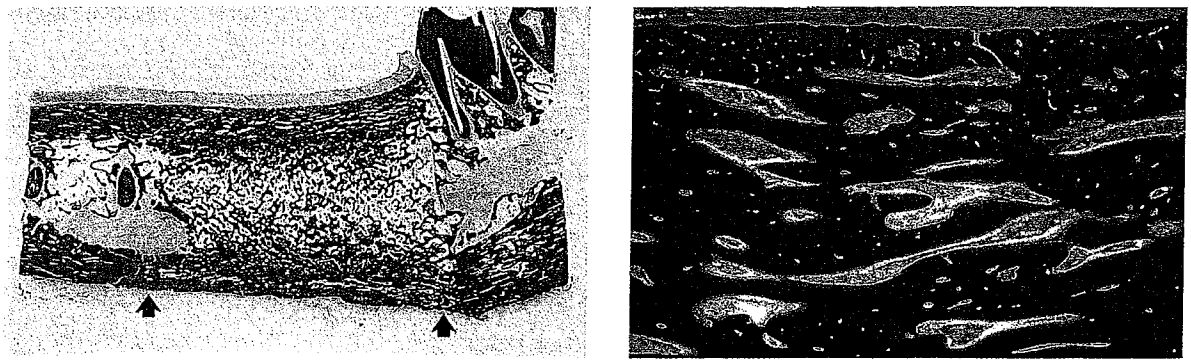


Fig 6.—Group 1 (6-month specimen). Left, Histologic section demonstrates mineralized bone formation across the entire defect. Mineralized bone (68.1% of tissue) represented by black-stained area. Arrows mark edges of the defect (modified von Kossa's stain, original magnification ×0.5). Right, New bone formation demonstrating multiple lacunae (modified von Kossa's stain, original magnification ×25).

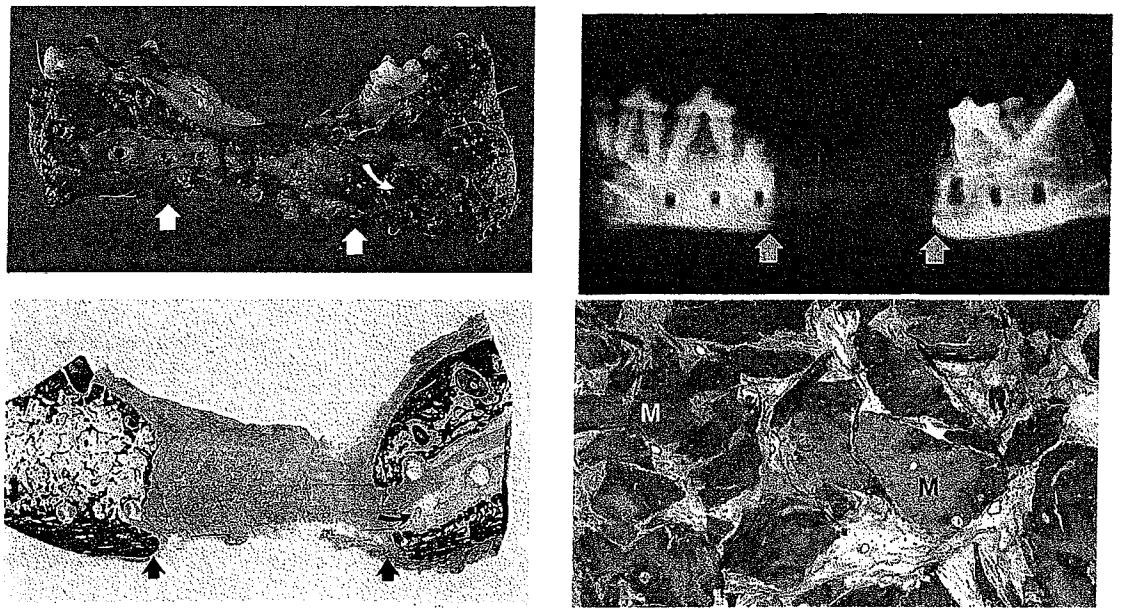


Fig 7.—Group 2 (3-month specimen). Top left, Harvested reconstructed segment shows soft tissue bridging the defect. Note the bone erosion where the plate contacted the bone surrounding the mandibular defect (curved arrow). Arrows point to edges of the defect. Top right, Roentgenogram demonstrates absence of bone formation. Note the soft tissue bridging the defect. Arrows mark edges of defect. Bottom left, Histologic section reveals fibrous tissue bridging defect with no bone formation. Curved arrow points to region of bone resorption. Arrows point to edges of defect (modified von Kossa's stain, original magnification ×0.5). Bottom right, Note the islands of residual bone matrix and no bone cell activity. M indicates bone matrix (modified von Kossa's stain, original magnification ×25).

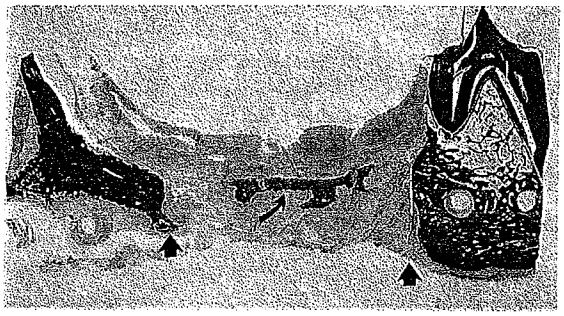


Fig 8.—Group 2 (6-month specimen). Histologic section reveals spotty bone formation (curved arrow) within fibrous tissue bridging 3-cm defect. Arrows point to edges of defect (modified von Kossa's stain, original magnification ×0.5).

Table 2.—Percentage Area of Defect Replaced by Mineralized Bone

Group	Type of Implant*	Mean Percentage Area of Tissue Occupying the Defect That Was Replaced by Mineralized Bone, mo	
		3	6
1	Inactive dog bone matrix carrier *with* BMP-2 (test)	50	68
2	Inactive dog bone matrix carrier *without* BMP-2 (control implant)	0	4
3	No implant (control)	0	0

*BMP-2 indicates bone morphogenetic protein-2.

Table 3.—Results of Biomechanical Testing

Group		Mean Maximum Moment (N − m)		Mean Bending Strength Expressed as a Percentage of Contralateral Hemimandible
		Site of Reconstruction	Contralateral Hemimandible	
1	3 mo	7.4	81.32	9
	6 mo	22.6	83.95	27
2	3 mo	0.0	69.36	0
	6 mo	0.0	69.19	0

the gingiva to allow healing of the intraoral defect before mandibular reconstruction. Therefore, a segmental mandibular defect could be created through an extraoral incision and reconstructed without intraoral contamination. With no intraoral defect, animals could be begin oral feedings soon after reconstruction. This animal model has several drawbacks. The healing period after tooth extraction delays the reconstruction for several weeks, increasing the cost of daily animal care. In the clinical setting, patients undergoing major mandibular reconstruction after ablative cancer surgery or trauma usually require closure of intraoral defects at the time of reconstruction. Removal of teeth in a first stage operation to avoid an intraoral suture line does not simulate the true clinical setting.

To prevent extensive intraoral contamination after mandibular reconstruction, patients are usually fed via a feeding tube for 7 to 14 days. In this study, an esophagostomy was created to simulate the clinical situation after mandibular reconstruction, allowing early feeding of the animals without gross intraoral contamination. Dogs do not tolerate feeding tubes, so the best option was to create a feeding esophagostomy. Not only did the esophagostomy allow administration of a complete liquid diet on the third day after reconstruction, but the intraoral suture line was also exposed to the normal intraoral contamination (saliva) that would be encountered in the clinical setting. Exposure of the suture line to saliva permitted evaluation of the infection rate of BMP-2 implants in a clean but not sterile *(clean-contaminated)* surgical field. After 14 days, the animals were given a soft diet by mouth with minimal food leakage out of the esophagostomy. By 14 days, the intraoral suture lines healed well, with little chance of mandibular defect site contamination with food particles. Furthermore, by day 14 the BMP-2 implants had a chance to form bone to provide some structural integrity at the site of reconstruction.

In this study, no infections of any of the group 1 implants (carrier with BMP-2) developed, despite implantation in a clean-contaminated surgical field and primary closure of 3- to 4-cm intraoral gingival defects. However, local infections of the implants developed in three group 2 animals (carrier without BMP-2) that required antibiotic therapy. The absence of infection in the BMP-2 implants may be due to the chemotactic response initiated by the BMP-2 implant. Increased influx of inflammatory cells and rapid vascularization of the implant could act to decrease the incidence of infection. A low infection rate would make these implants much more attractive for clinical use.

The mandibles reconstructed with BMP-2 implants were functionally stable, as demonstrated by the ability of the dogs to tolerate a solid diet orally after removal of the reconstruction plates at 10 weeks. It may have been possible to remove the plates earlier, but pilot data revealed that plate removal at 10 weeks allowed sufficient bone formation to provide functional stability of the reconstructed segments. Furthermore, plate removal probably increased bone remodeling by exerting forces of mastication on the reconstructed segment. Lack of bone formation and gross instability of the reconstructed segments of animals in groups 2 and 3 (controls) precluded plate removal until these dogs were killed.

In dogs, the premolars are used for grasping and the molars are used for masticating, with the greatest bite forces exerted on the first molar. In this study, the first molar was removed at the site of the defect. Therefore, the animals may have chewed on the opposite side of their mouth, directing maximum bite forces on the nonoperated hemimandible. The mandibular defects reconstructed with BMP-2 demonstrated sufficient strength to support the forces of mastication, which translated into a functionally stable reconstruction. However, a more accurate determination of the maximum bite forces that could be supported by the reconstructed segment would require osseointegration of a dental implant into the new bone. This would permit the animal to chew on the side of the reconstruction, exerting maximum bite forces on the reconstructed segment. Biomechanical testing was performed to provide direct quantitative testing of the bending strength of the reconstructed segments.

The mature portion of BMP-2 is highly conserved among species, which would indicate few changes between human and canine BMP-2.[19] Immunoreactivity studies revealed no antibodies to human recombinant BMP-2 in any of the animals in this study. The absence of circulating antibodies suggests that (1) the dose used was too low to elicit an antibody response, (2)

the method of delivering BMP-2 prevented antibody formation, or (3) canine BMP-2 is highly conserved to human BMP-2.

Histologic findings revealed bone growth across the mandibular defects in the 3- and 6-month animals who underwent reconstruction with BMP-2 implants. The cellular morphologic appearance of the osteoblasts was indicative of active bone formation. The BMP-2 implants probably stimulated the rapid influx and transformation of primitive mesenchymal cells into osteoblasts, resulting in extensive host bone formation across the mandibular defects.[12] The reconstructed segments may have undergone endochondral bone formation, initially forming cartilage that eventually underwent mineralization, but earlier time points would be needed to verify this.

Despite the extent of bone formation, no evidence of bone formation was found beyond the boundaries of the defect. This process of induced bone formation can be rationalized as a controlled response to highly concentrated (supraphysiologic) levels of the bone-inducing protein, BMP-2, that is also present in host tissue but at a much lower concentration. At 6 months, the morphologic appearance and volume density (mineralization) of the new bone approached that of the normal bone surrounding the defect with no evidence of bone resorption. Osseointegration of the reconstructed segment was complete to the point where the interface between the normal bone surrounding the defect and the bone-inducing implant was difficult to identify.

In the group 2 and 3 control animals, there were complete defects at the site of reconstruction that were occupied by fibrous scar or callous formation. The edges of the defect demonstrated a cellular morphologic appearance indicative of low bone cell activity. The lack of bone formation in the control animals demonstrates the inability of the host to induce any significant bone formation across the 3-cm defects in the absence of periosteum. The small islands of bone that did form were along the margins of the gingival mucosa where small segments of periosteum may have been left behind.

Quantitative biomechanical testing (three-point bend testing) revealed relatively low biomechanical strength of the mandibular defects reconstructed with BMP-2 (group 1) compared with the contralateral nonoperated hemimandible. However, the bending strength increased dramatically from the 3-month (7.4 N-m) to the 6-month (22.6 N-m) specimens. The mean percentage of the tissue occupying the defects that was replaced by mineralized bone correlated with increases in biomechanical strength (failure patterns), as demonstrated by the increase in mineralization values of the 6-month specimens (68% mineralization, mean) compared with the 3-month specimens (50% mineralization, mean). This significant correlation, however, was not a direct relationship. Furthermore, the degree of plasticity of the group 1 reconstructed segments decreased with increasing mineralization. These data indicate that the bending strength of the mandibular defects reconstructed with BMP-2 may have been, in part, related to the degree of mineralization and may increase with time. Other factors that may influence biomechanical strength are the architecture of the newly formed bone and the character of the bone after remodeling.

The mean width of the new bone occupying the defects was only 62% of the mean width of the contralateral, nonoperated hemimandibles, even though the contour of the reconstructed segments closely approximated the contour of the normal bone surrounding the mandibular defect. This difference in width in the transverse plane was, in part, due to the increased width of the contralateral, nonoperated hemimandible in the region of the first molar. The fact that maximal bite forces are applied to the first molar may account for the increased width of that region of the normal, nonoperated canine mandible.

The bone matrix carrier lacked structural stability and was probably compressed by the walls of the defect, resulting in formation of bone that was thinner in the transverse plane. Biomechanical testing was performed in the transverse plane with the lingual surface in tension. Therefore, the reconstructed segments were tested at its weakest point where the bone was thinner and then compared with the widest segment of the contralateral hemimandible in the region of the first molar. This critical comparison is important, however, because the mandible should be reconstructed in a manner that exhibits biomechanical strength comparable with the normal mandible to protect against fracture due to maximum bite forces and blunt trauma. Use of a moldable, less-deforming carrier or a mandibular tray that would prevent compression of the BMP-2 implant could result in new bone that would better match the width, and possibly approach the biomechanical strength of the contralateral hemimandible.

The mandible is one of the strongest bones in the body, requiring high forces to elicit a fracture. Because no other studies have been published that report three-point bend testing on a mandible reconstructed with a bone-inducing implant, the relatively low bending strength of the reconstructed segments may actually be comparable with other methods of reconstruction. One of many presently used methods of mandibular reconstruction include stabilizing mandibular defects with a stainless steel or titanium reconstruction plate with or without a bone graft.[20,21] In many cases, the reconstruction plate alone provides the support across the defect because (1) no bone graft was used, (2) insufficient bone was used, (3) the bone graft failed, or (4) the bone graft lacked sufficient strength to support the forces of mastication.

To provide clinically relevant comparative baseline data, the biomechanical strength of 3-cm full-thickness mandibular defects bridged only by stainless steel or titanium reconstruction plates was determined. Assuming the cortical screws and plates become integrated with the proximal and distal edges of the defects, the segment of reconstruction plate bridging the defect should represent the strength of the reconstruction. The biomechanical strength of the mandibular defects reconstructed with BMP-2 implants (6-month animals) demonstrated significantly greater biomechanical strength than did the hemimandibles reconstructed with only a stainless steel reconstruction plate. The bending

strength of the 6-month group 1 mandibular defects reconstructed with BMP-2 implants was also greater than the bending strength of the titanium plate reconstructions. The biomechanical strength of mandibles reconstructed with BMP-2 must be compared with other presently used clinical methods (such as autologous bone grafts) to help determine what degree of biomechanical strength can be considered acceptable if the strength of nonoperated, normal mandibles cannot be matched.

Connole et al[22] stated that the goals of mandibular reconstruction should include (1) establishment of mandibular continuity, (2) establishment of an osseous alveolar base for a prosthesis, and (3) correction of alveolar and soft-tissue deficiencies in preparation for prosthetic reconstruction. In this study, a canine model was used to demonstrate how biocompatible, easy to use, BMP-2 bone-inducing implants can restore mandibular continuity, establish a functionally stable osseous alveolar base that could accept a prosthesis (such as dentures), and provide acceptable masticatory function. A major advantage to this method of reconstruction is that mandibular continuity could be established without the morbidity associated with the defect at a bone graft donor site. Other advantages include the ability to mold the bone-inducing implant to reconstruct a complex region of the mandible (anterior arch, angle, and ramus). Future studies will involve the insertion of osseointegrated dental implants into the reconstructed segment to evaluate the ability of the new bone to support maximal bite forces and eventually provide complete oral rehabilitation.

Of great importance to this study is the fact that BMP-2 is a human recombinant protein that is free of contaminating proteins and can be used without the risk of transmitting any infectious diseases. Commercial production of BMP-2 using recombinant DNA technology could provide a high-quality, uniform product with reproducible activity that could be manufactured in large quantities and sold at a reasonable cost to the patient.

Further studies are necessary to answer other questions about BMP-2 before its clinical use. Other carrier materials for BMP-2 that are completely biocompatible and bioerodible need to be investigated. In this study, compression of the implants in the transverse plane resulted in decreased width of the bone bridging the defect. This bone exhibited relatively low biomechanical strength, in part due to the decreased width of the new bone in comparison with the contralateral nonoperated hemimandible. This problem appears to be due to a deficiency in the carrier and not the bone-inducing capability of BMP-2.

Polymers such as polylactic acid, polyglycolic acid, or combinations thereof may prove to be effective carrier materials because they are bioerodible, can be formulated to take on many different degrees of solidity, and have a low degree of tissue reactivity. Synthetic polymer carriers would be far superior to a bone matrix carrier, which is an allograft and is not ideal for clinical use. Furthermore, polymers used in the form of a resorbable tray or formulated into a nondeformable, semirigid carrier material for BMP-2 could induce bone formation to reconstruct a bony defect to its exact dimensions with increased biomechanical strength.

If BMP-2 implants are to be used to reconstruct defects created after ablative cancer surgery, this factor's effectiveness in radiated tissues must be evaluated. Studies are presently being conducted to evaluate the effectiveness of BMP-2 in radiated tissues exhibiting both acute and chronic radiation injury. Ultimately, clinical trials of BMP-2 will determine the actual clinical applicability of these bone-inducing implants.

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We are grateful to Synthes Maxillofacial, Paoli, Pa, for providing the reconstruction plates, screws, and instrumentation necessary to rigidly fixate the mandibular defects.

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Induction of Dentin Formation on Canine Amputated Pulp by Recombinant Human Bone Morphogenetic Proteins (BMP)-2 and -4

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Abstract. Dental pulp cells have the potential to differentiate into odontoblasts. The molecular mechanisms underlying differentiation are not clear. Demineralized dentin matrix is osteoinductive and contains bone morphogenetic protein (BMP) activity. BMPs have been implicated in embryonic odontogenic differentiation and hence may play a role in the differentiation of adult pulp cells into odontoblasts during pulpal healing. This study examined the hypothesis that BMPs induce dentin formation on amputated canine pulp. Recombinant human BMP-2 and BMP-4 were capped with inactivated dentin matrix on amputated pulp. At two months, the amputated pulp was filled with tubular dentin in the lower part and osteodentin in the upper part. The amount of dentin formed was markedly diminished when dentin matrix alone was implanted. These findings imply that recombinant human BMP-2 and BMP-4 induce differentiation of adult pulp cells into odontoblasts. Thus, BMPs may have a role in dentistry as a bioactive pulp-capping agent to induce dentin formation.

Key words. Bone Morphogenetic Proteins-2 and -4, Dentin, Dental Pulp, Odontoblasts, Differentiation, Dental Pulp Capping, Pulpotomy.

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Introduction

Dental pulp tissue retains considerable potential for regeneration and repair (Fitzgerald, 1979; for a review, see Yamamura, 1985). The precise cellular and molecular mechanisms underlying this functional repair are, however, still unknown. Similarly, bone is also capable of complete repair. The molecular basis of bone repair has now been attributed to a family of bone morphogenetic proteins (BMPs) based on *de novo* induction of bone into extraskeletal sites. Eight BMPs—namely, BMP-1-BMP-7 and osteogenic protein (OP)-2—have so far been cloned and expressed (Wozney *et al.*, 1988; Celeste *et al.*, 1990; Özkaynak *et al.*, 1990; Wang *et al.*, 1990; Hammonds *et al.*, 1991; Özkaynak *et al.*, 1992; for a review, see Reddi, 1992). All BMPs except BMP-1 belong to the transforming growth factor-beta (TGF-β) superfamily, a large group of signaling proteins with multiple biological activities controlling the differentiation of a variety of cell types (Rizzino, 1988). A BMP-like activity which induces bone formation is also present in dentin matrix (Butler *et al.*, 1977; Conover and Urist, 1982; Katz and Reddi, 1988; Mera, 1988; Kawai and Urist, 1989; Bessho *et al.*, 1990, 1991). The precise BMPs in dentin have not been identified. It is likely that BMPs may play a role in dentinogenesis, and in fact Lyons *et al.* (1990) have shown that BMP-2 mRNA expression is evident in dental papilla and odontoblasts in developing tooth bud. In organ culture of dental papilla from 17-day-old mouse embryos, BMP-2 stimulates matrix secretion. When combined with inactive total EDTA-soluble dentin proteins, BMP-2 stimulates odontoblast differentiation (Bègue-Kirn *et al.*, 1992). Recently, it has been shown that BMP-4 mRNA expression is first detected in the presumptive dental epithelium and is shifted thereafter to the condensed dental mesenchyme. The message is then restricted to the pre-odontoblasts during bell stage and is

Figure 1. BMP-2 with carrier, after 2 months. Note the formation of the thick in-duced dentin consisting of tubular dentin and osteodentin in the lower and middle parts of the cavity on the amputated (arrows) pulp. In the upper part, pieces of implanted dentin matrix surrounded by pulp tissue can be seen.

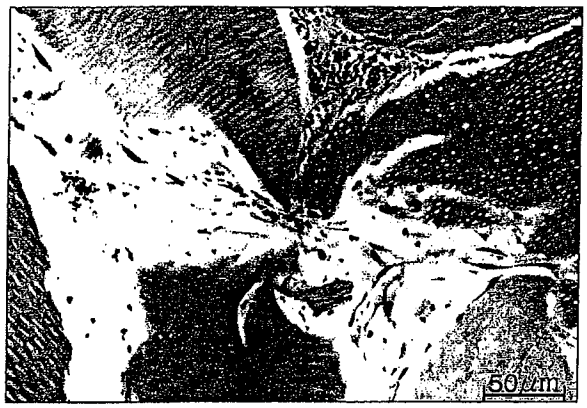

Figure 2. BMP-2 with carrier, the superior part of the cavity. Red blood cells and spindle-shaped cells can be seen around implanted dentin matrix (M).

correlated with tooth induction (Vainio *et al.*, 1993). BMP-2 mRNA expression is first seen in the inner enamel epithelium about 3 days later than BMP-4. Then it is shifted to the mesenchymal cells of dental papilla and appears in pre-odontoblasts during their terminal differentiation, suggesting a role in odontoblast differentiation. Recombinant BMP-2 or BMP-4 containing agarose beads is able to induce BMP-4 expression in the mesenchyme in the absence of epithelium, suggesting that BMP-4 is involved in the biochemical pathway mediating early inductive interactions between the epithelial and mesenchymal tissues (Vainio *et al.*, 1993). In view of this, BMPs play an integral role in dentin development and morphogenesis.

Using bovine pulp cell cultures, we have shown that BMP-4 mRNA is expressed on day 14 during the increased expression of the extracellular matrix protein (Nakashima *et al.*, 1994). BMP-2 mRNA is expressed on day 28, when pulp cells have already differentiated into pre-odontoblasts. Genes for BMP-2 have been cloned from the cells in pulp cell culture on day 28 (Nakashima, unpublished data). *In vitro*, recombinant human BMP-2 increased osteocalcin synthesis, BMP-4 increased expression of α1(I) collagen mRNA, and both BMP-2 and BMP-4 stimulated alkaline phosphatase activity. These findings suggest a regulatory role for BMPs on the differentiation of pulp cells into pre-odontoblasts (Nakashima et al., 1994). Partially purified BMP from bone has been shown to induce dentin on the amputated pulp (Nakashima, 1990b). It is possible that BMP-2 and BMP-4 mRNA may be expressed in pulp mesenchymal cells during pulpal wound healing and resultant formation of dentin.

Bio-active pulp-capping agents need to be developed. Agents that enhance healing potential of pulp tissue and induce the formation of a large amount of dentin over exposed pulp which protects from microleakage and pulp infection could be clinically useful for direct pulp capping. In this communication, we have examined the hypothesis that BMPs in dentin matrix may induce dentin formation. The results revealed that BMPs may be used as potent bio-active pulp-capping agents.

Materials and methods

Preparation of samples

Inactivated demineralized dentin matrix powder with a Guanidine-hydrochloride-extracted particle (200-500 μm in size) was prepared from bovine permanent incisors as previously described (Nakashima, 1989). Two μg of recombinant human BMP-2 (mol wt, 30,000) and recombinant human BMP-4 (mol wt, 32,000) were added, respectively, to carriers: 10 mg of inactivated dentin matrix powder, 0.5 mg of chondroitin 6-sulfate sodium salt (Seikagaku Kogyo Co., Nagoya, Japan), and 250 μg of acid-soluble type I rat tail tendon collagen (Muthukumaran *et al.*, 1988). The samples were mixed and left for 1 h at room temperature before the proteins were dried under vacuum.

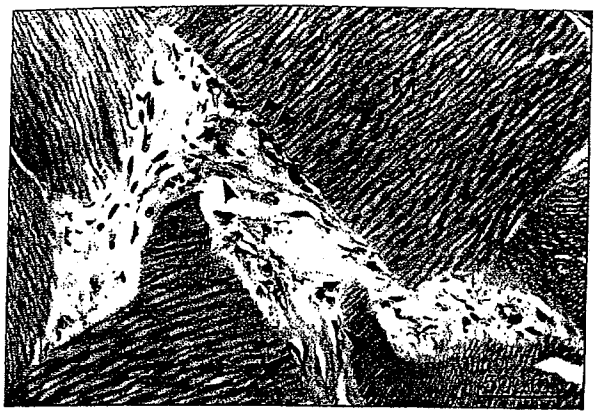

Figure 3. BMP-2 with carrier, the upper part. Both undifferentiated large cells (arrowheads), around implanted dentin matrix (M) and attached to matrix (M), and spindle-shaped cells (arrows) synthesizing matrix around them can be seen.

Pulp-capping procedures

Twelve teeth from 2 young adult dogs weighing 15 kg were used. Surgical anesthesia was obtained by intravenous injection of 20 mg pentobarbital sodium *per* kg of body weight. The pulps of the upper third incisor and canine and of the lower canine were amputated as described previously (Nakashima, 1989). BMP-2 with carrier, BMP-4 with carrier, and carrier alone were capped into the cavities of four teeth on the amputated pulp. The dry samples were moistened with saline and transferred to the amputated pulp by a small brush. The cavities over the implanted materials were filled with Elite® cement (zinc phosphate cement, G.C. Dental Industrial Corporation, Tokyo, Japan) and Clearfil® (resin composite, Kurare Company, Okayama, Japan).

Histological examination

All of the animals were killed after 2 months. The apical two-thirds of the root was immediately removed, and the teeth were fixed in 10% formalin. They were then demineralized in 10% formic acid, embedded in paraffin, sectioned at 4-5 μm, and stained with hematoxylin and eosin for routine light microscopy. The extracted teeth were evaluated by contact radiography by soft-x-ray apparatus, type SRO-M50 (Sofron Company Limited, Tokyo), at 40kV, 5mA, for 1 min before demineralization.

Quantitative analyses of newly formed dentin

We examined relative amounts of newly induced dentin in each sample by capturing, through the microscope, video images of the histological preparations, 5 sections from one tooth, at 150-μm intervals. Image Grabber 1.15® (Neoteck Limited, Eastleigh, Hampshire, UK), installed in a Macintosh Quadra 700 (Apple Computer, Cupertino, CA, USA) connected to the video camera, was used to digitize the images. Using Canvas 3-J® software (Deneba Systems Inc., Miami, FL, USA), we hand-traced the on-screen image outlines of pulp tissue and implanted dentin matrix in the cavity on the amputated pulp, and the total areas of these were determined by Ultimage/24® software (Image & Measurement Inc., Mirmande, France).

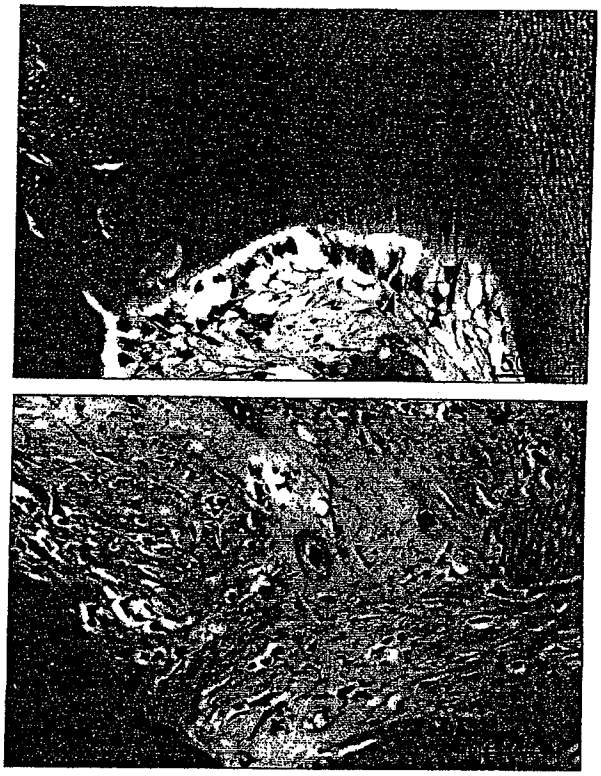

Figure 4. BMP-2 with carrier, the middle part. (a, top) Note the odontoblast-like cells (arrowheads) extending their cytoplasmic processes into newly formed tubular dentin (TD). (b, bottom) Note osteodentinocytes (arrows) entrapped in osteodentin.

Results

Histological observations

Two months after implantation of BMP-2 with carrier, there is a gradient of cellular response from the bottom of the cavity on the amputated pulp to the top (Fig. 1). The cavity on the amputated pulp was divided into four parts—designated lower, middle, upper, and superior— at 1-mm increments from the amputated site. In the superior part of the cavity, red blood cells, monocytes, and spindle-shaped mesenchymal cells with slender processes containing elongated rod-shaped nuclei were observed (Fig. 2). The upper part of the cavity was filled with the pulp tissue and consisted of loose connective tissue containing capillaries, spindle-shaped cells, and undifferentiated large, oval, or polygonal cells whose nuclei were weakly stained. Some of

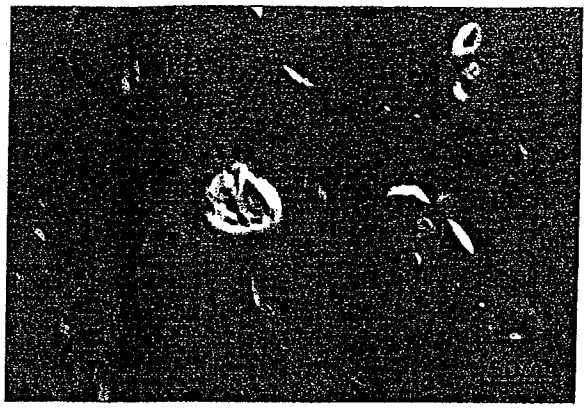

Figure 5. BMP-2 with carrier, the lower part. A few odontoblasts and a capillary remain in tubular dentin (TD).

Figure 6. BMP-4 with carrier, after 2 months. Note a large amount of induced dentin consisting mainly of tubular dentin in the lower part and osteodentin in the upper part. Remaining pulp tissue under the amputated site (arrows) is normal.

the large cells attached to the implanted dentin matrix, and some of the spindle-shaped cells formed matrix around them (Fig. 3). In the middle part of the cavity, regularly arranged odontoblast-like, polarized cells with long cytoplasmic processes had formed irregular tubular dentin (Fig. 4a). Darkly stained osteodentinocyte-like cells with round or oval nuclei were embedded in irregular oval lacunae, synthesizing extracellular mineralized matrix, osteodentin (Yamamura, 1985) (Fig. 4b). The lower part of the cavity was filled mostly with irregular tubular dentin, and a few odontoblasts, osteodentinocytes, and capillaries remained in the mineralized matrix (Fig. 5). Some of implanted dentin matrix was resorbed.

Teeth capped with BMP-4 plus carrier showed histological results similar to those of BMP-2 plus carrier, as described above (Fig. 6). Considerable amounts of osteodentin surrounded osteodentinocyte-like cells (Fig. 7a). Occasionally, tubular dentin was also seen adjacent to the osteodentin (Fig. 7b). In the superior part of the cavity, spindle-shaped cells deposited extracellular matrix around the cells themselves (Fig. 8).

In teeth which were capped with carrier alone, all parts of the cavity were filled with pulp tissue (Fig. 9). Tubular dentin formation was minuscule compared with BMP-2- and BMP-4-enriched carrier, although odontoblast-like cells lined the implanted matrix in the lower and middle parts of the cavity (Fig. 10). Osteodentin formation was rarely seen. In all implanted teeth, there was no sign of inflammation or dystrophic mineralization in the remaining pulp tissue. Some of the newly induced dentin appeared in discontinuous zones.

Radiography

Contact-radiographic observation (Fig. 11) showed that optimal mineralization had occurred in the cavities in teeth implanted with BMP-2 and BMP-4. When carrier alone was implanted, there was a significantly larger radiolucent area compared with that when BMP-2 and BMP-4 were capped.

Quantitative analyses

Since there was no sign of inflammation, the area excluding pulp tissue and implanted dentin matrix was regarded as newly formed dentin. Therefore, the pulp tissue and implanted dentin matrix were traced in definite area from lower and middle parts of the cavity on the amputated pulp, and the relative areas of newly formed dentin were calculated as follows: [1-(total area of pulp tissue and implanted matrix)/definite area)] x 100%. The percent relative area of BMP-2- and BMP-4-enriched carrier was 80-82% compared with 42% in carrier alone (Table 1).

Discussion

This investigation has demonstrated that both recombinant human BMP-2 and BMP-4 plus dentin matrix carrier induced the formation of a large amount of dentin. The newly induced dentin was mostly tubular dentin in the lower part of the cavity and osteodentin in the upper part. There was a graded cellular response from the bottom of the cavity on the amputated pulp to the top, as in previous observations from 1 week to 2 months post-operatively (Nakashima, 1989).

"Reparative dentin" is defined as a localized zone of dentin that is deposited in non-exposed pulps in response to some external stimulation (Cox *et al.*, 1992). Deposition of

Table. Relative areas of induced dentin in response to BMPs

Implanted Materials	Percent Relative Areas Mean ± SD
Carrier alone	42.4 ± 4.4
BMP-2 with carrier	80.7 ± 4.1[a]
BMP-4 with carrier	82.1 ± 5.5[a]

[a] $P < 0.001$ (t test); n = 4 (mean of 5 sections used as one sample).

reparative dentin has been considered to be a consequence of either naturally occurring physiologic factors, such as occlusal attrition, erosion, abrasion, and aging, or pathologic effects, such as caries, periodontal disease, orofacial infections, and instrumentation trauma during tooth preparation (Beust, 1931). "Dentin bridge" has been used in the literature to describe that deposition of a new matrix either directly adjacent, or subjacent, to some sort of material, such as the pulp-capping agent. Cox *et al.* (1987, 1992) have shown, however, that healing of exposed pulp is dependent not on the effect of a particular type of medicament but rather on the capacity of the capping agents to prevent bacterial leakage. The inherent healing capacity of the pulp tissue has been suggested by several authors (Berman and Massler, 1958; Schröder and Granath, 1971; Schröder and Sundström, 1974; Yamamura, 1985). In this manuscript, I have chosen to describe BMP-2- or BMP-4-stimulated dentin as "induced dentin" formation. I wish to distinguish this from the terms "reparative dentin" formation and "dentin bridge" formation, used by restorative dentists such as Cox *et al.* (1992).

BMP-2 and BMP-4 do not affect proliferation of pulpal mesenchymal cells *in vitro* (Vainio *et al.*, 1993; Nakashima *et al.*, 1994), but stimulate differentiation of pulp cells into odontoblasts *in vitro* (Bèque-Kirn *et al.*, 1992; Nakashima *et al.*, 1994). BMP-2 and BMP-4 induce the expression of *Msx-1* and *Msx-2* genes, which function as transcription factors controlling the transcription of other genes, suggesting the widespread signaling functions of BMP-2 and BMP-4 in morphogenesis and organogenesis (Vainio *et al.*, 1993). Albumin does not have any effect on proliferation and differentiation of pulp cells *in vitro* (Nakashima, 1992). The cavity on the amputated pulp was filled with pulp tissue, with little or no induced dentin *in vivo* in response to albumin placement (Nakashima, 1990b). The amount of newly induced dentin was much smaller in teeth with inactivated dentin matrix than in teeth with BMP-2- and BMP-4-enriched carrier (Table 1). BMP-2 with collagen carrier induced dentin in a dose-dependent manner (unpublished data). These findings suggest that BMP has a stimulatory effect on dentin formation. It is clear that the cavity was free from bacterial infection due to aseptic instrumentation, capping, and double-sealing with zinc phosphate cement and Clearfil®, with enamel etching and

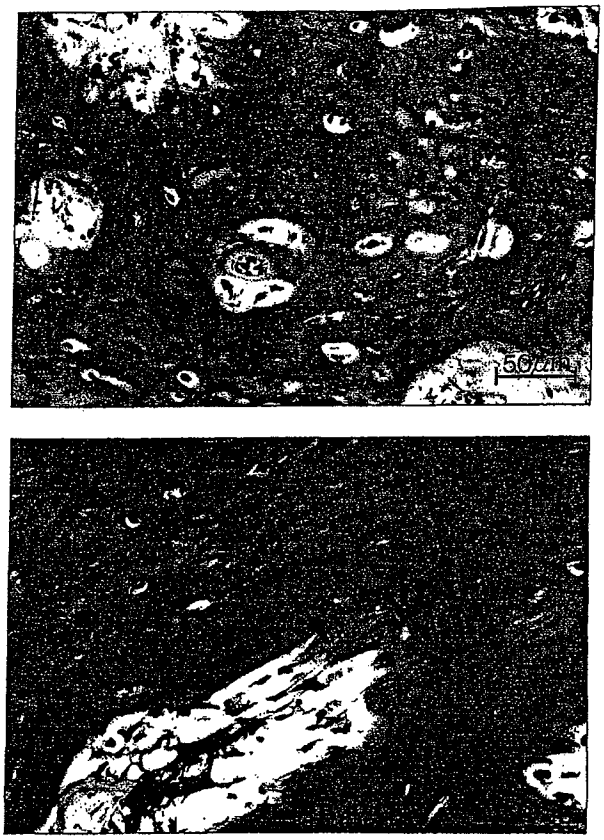

Figure 7. BMP-4 with carrier, the upper part. (a, top) Representing osteodentin. Many osteodentinocytes entrapped by mineralized matrix within irregular oval lacunae and capillaries can be seen. (b, bottom) Representing tubular dentin (TD) surrounded by osteodentin (OD). Cytoplasmic process (arrow) of odontoblast-like cell extending into tubular dentin (TD).

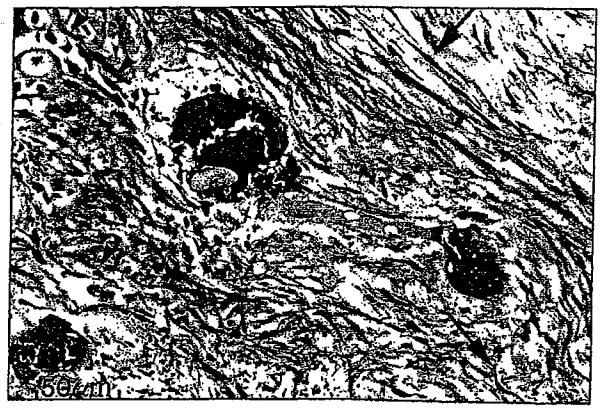

Figure 8. BMP-4 with carrier, the superior part. Note spindle-shaped cells (arrows) and osteodentinoblasts (arrowheads) synthesizing extracellular matrix.

Figure 9. Carrier alone, after 2 months. Note much smaller amount of induced dentin compared with that in Figs. 1 and 6.

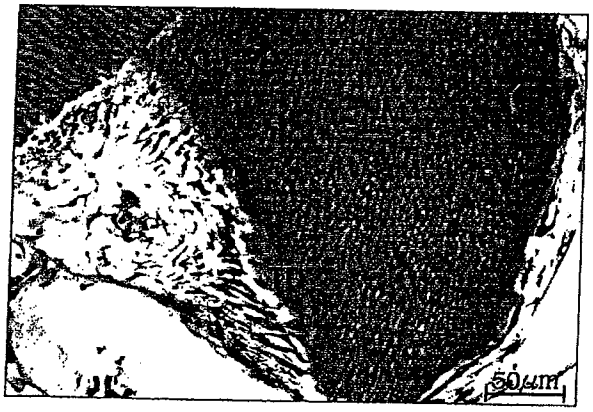

Figure 10. Carrier alone, the middle part. Note odontoblast-like cells lining implanted dentin matrix (M), forming a small amount of tubular dentin (TD).

bonding which covered the whole buccal face of the teeth. Therefore, the healing sequence in BMP-2 with carrier and BMP-4 with carrier implanted teeth is described as follows:

(1) Fibroblast-like cells migrate from underneath pulp tissue and proliferate (Fitzgerald, 1979), and pulp tissue regenerates on the amputated pulp under the proper environment without bacterial infection.

(2) Inactivated dentin matrix provides a local site for pulp cells suitable for attachment, providing an optimal environment for differentiation.

(3) BMP-2 and BMP-4 stimulate the differentiation of the attached cells into odontoblasts.

In teeth which were capped with BMP-2- or BMP-4-enriched carrier, the lower part of the cavity was filled mostly with tubular dentin. The implanted 4M-guanidine-extracted demineralized dentin matrix is inactive because it is devoid of any growth factors and osteogenic protein, as previously shown *in vitro* (Somerman *et al.*, 1987) and *in vivo* (Katz and Reddi, 1988). In teeth which were capped with carrier alone, tubular dentin formation was diminished compared with teeth implanted with BMP-2- and BMP-4-treated implants. A recent work using osteogenic protein-1 (BMP-7) reconstituted with type I collagen matrix from cortical bone for direct pulp-capping demonstrates not tubular dentin but osteodentin formation over exposed pulp (Rutherford *et al.*, 1993). The authors attribute this to its short-term application (6 weeks). The adhesion of pulp cells to an appropriate surface may be the critical requirement for the appearance of elongated, polarized, odontoblast-like cells (Veis, 1985). Biomechanical connection between the components of extracellular matrix and the intracellular cytoskeletal constituents of peripheral papilla cells is critically important in determining their mitogenesis, shape, and function (Ruch, 1990). An unmineralized matrix of pulpal ectomesenchymal origin synthesized either by original odontoblasts or by pulp tissue cells—such as fibrodentin, osteodentin, predentin, and demineralized dentin—is essential for the initiation of tubular dentin formation in pulpal healing (Baume, 1980; Yamamura, 1985; Nakashima, 1989, 1990a,b; Tziafas and Kolokuris, 1990; Tziafas *et al.*, 1992). The demineralized bone matrix is known to induce osteodentin formation on amputated pulp (Sluka *et al.*, 1979) and in pulp tissue (Tziafas and Kolokuris, 1990). Therefore, it is likely that the increased tubular dentin formation in the present study, in contrast to osteodentin formation in teeth which were implanted with BMP-7 with collagenous bone matrix (Rutherford *et al.*, 1993), might be due to the carrier, dentin matrix, rather than to the type of BMP used.

The present study demonstrated the utility of recombinant human BMP-2 and BMP-4 as bio-active pulp-capping agents. These morphogenetic factors can induce a large amount of dentin on amputated pulp without affecting the remaining pulp. Further refinement of the delivery system may result in predictable optimal dentin induction.

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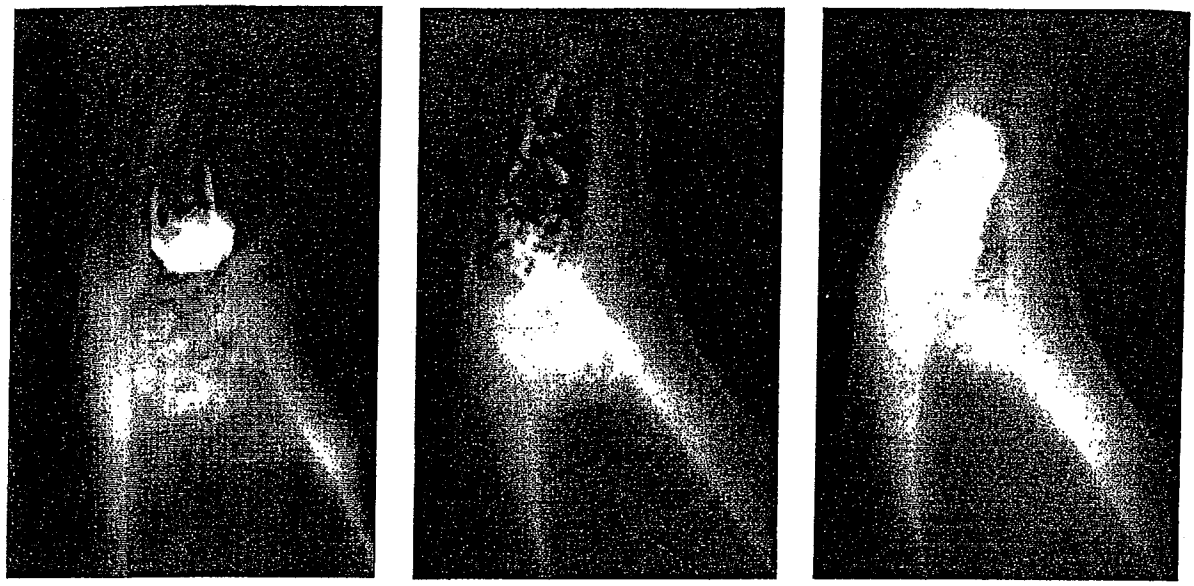

Figure 11. Contact radiographs after 2 months, showing mineralization of induced dentin much more diffuse and porous with carrier alone (a, left) compared with BMP-2 with carrier (b, center) and BMP-4 with carrier (c, right). Bars represent 2 mm.

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